DEVELOPMENT OF NEW DEBRIS MEDIATED HUMAN OSTEOLYSIS MODEL TO INVESTIGATE THE POTENTIAL THERAPEUTIC ROLE OF MATRIX METALLOPROTEINASE INHIBITORS IN ASEPTIC LOOSENING

Thesis submitted for the degree of Doctor of Medicine at the University of Leicester

by

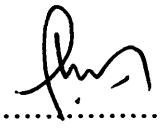
Shong Meng Ong

University of Leicester

2003

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.



.....

Shong Meng Ong

October 2003

Acknowledgments

This thesis would not have been possible without the overwhelming support and guidance of my teacher Mr Grahame John Saint Clair Taylor.

I also want to thank my wife Jan Ling for her unlimited patience, understanding, and encouragement.

Prizes, publications & presentations

1) Doxycycline inhibits bone resorption by human interface membrane cells from aseptically loose hip replacements.

   SM Ong, GJS Taylor

   J Bone Joint Surg [Br] 2003; 85-B:456-61.

   Won the Pfizer academic travel awards 2002.

2) Can NTx be used as an in vitro marker of wear particulate induced osteolysis.

   SM Ong, GJS Taylor

   J Bone Joint Surg [Br] 2003; 85-B: Supp 1:8

   Awarded best presentation for young researcher (clinician) at the British Research Orthopaedic Society (BORS) meeting 2001.

3) Inhibitions of debris mediated osteolysis by fluvastatin.

   SM Ong, GJS Taylor

   Presented at the British Orthopaedic Research Socity meeting 2003. (Short listed for best presentation for young researcher)

   Won the Smith & Nephew Research & Travel Awards 2004 (4^{th} prize).

Abbrevations

BCP	Bone Collagen Equivalents
BMP	Bone Morphogenetic Protein
CoCr	Cobalt Chrome
CTx	Crosslinked C-telopeptides
Dpy/Dpyr	Deoxypyridinoline
EGF	Epidermal growth factor
EHDP	Disodium ethane 1, 1-diphosphonate
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
GGHyl	Glucosylgalactosylhydoxylysine
GHyl	Galactosylhydroxylysine
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
HPLC	High-performance liquid chromatography
ICTP	I-C-telopeptide assay
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-11	Interleukin-11
M-CSF	Macrophage colony stimulating factor
MEM	Minimal essential medium
MMP	Matrix metalloproteinase
NTx	Cross-linked N-telopeptides

OA	Osteoarthritis
PBS	Phosphate buffer saline
PDGF	Platelet derived growth factor
PE	Polyethylene
PGE_2	Prostaglandin E_2
PMMA	Polymethylmethacrylate
PP	Pyrophosphate
Pyr	Pyridinoline
RANKL	Receptor activator N-Kappa ligand
TGF	Transforming growth factor
Ti	Titanium
TNF	Tumour necrosis factor
TRAP	Tartrate-resistant acid phosphatase

Index

CHAPTER 1

INTRODUCTION AND AIMS OF THE STUDY

1.1 INTRODUCTION

Total joint arthroplasty has evolved since its popularisation in 1959 by the late Sir John Charnley (1). Osteolysis and aseptic loosening remain the most common and important long term complications following total joint replacement (2). Aseptic loosening is characterised by bone destruction around the implant interface causing prosthesis loosening and bone loss. At present this requires complex revision surgery. The incidence of aseptic loosening increases with time, limiting the longevity of the implant.

It appears to be fuelled by the biological reaction to particulate wear debris and is likely to depend on several factors. These include

a) the type, amount and rate of wear debris production (3)

b) the access of this debris to the bone-implant interface (4)

c) the biological reaction to the wear debris which varies from person to person (5).

Efforts to minimise this problem target several areas of research such as minimisation of wear debris by looking for alternative bearing surfaces and development of new biologically inert material. Despite technologic advances, it is unlikely that wear debris will be completely eliminated. The alternative strategy is modulation of the biological response to particles by pharmacological means which aim to inhibit periprosthetic osteolysis.

Developing medical treatment for aseptic loosening will not only require a better understanding of the biological process of aseptic loosening but also the development of an effective laboratory model to test the potential of these drugs. An optimal model should allow rapid multiple testing, be cost effective and simple. This thesis concentrates on the development of a new in vitro particle induced osteolysis model and the testing of potential pharmacological agents such as doxycycline and statins against this process.

1.2 OBJECTIVES

The objectives of this study were to:

1. Investigate the ability of human interface membrane cells taken from aseptically loosened hip joints to resorb bone using an established radiolabelled murine calvaria model.
2. Investigate whether N-Telopeptides (NTx) can be used as an osteolysis marker instead of radioactive calcium for the investigation of aseptic loosening.
3. Develop a laboratory model of aseptic loosening using powdered human bone and NTx as an osteolysis marker.
4. Investigate the ability of doxycycline to inhibit debris mediated osteolysis.
5. Investigate the ability of statins to inhibit debris mediated osteolysis.

The study has been divided into a number of experiments. The first experiment was to demonstrate that cells from the interface membrane of aseptically loosened hips have the ability to resorb bone using the established radiolabelled murine calvaria model and at the same time to show that NTx can be used in this model as a resorption marker.

The second experiment involved the development of a new in vitro debris mediated osteolysis model using human bone instead of radiolabelled murine calvaria and again using NTx as the bone resorption marker.

The third and fourth experiments involved exploring the possibilities of doxycycline and statins for the potential treatment of aseptic loosening.

CHAPTER 2

CURRENT KNOWLEDGE OF ASEPTIC LOOSENING OF TOTAL JOINT REPLACEMENTS

(EPIDEMIOLOGY, AETIOLOGY & BIOLOGY)

2.1 EPIDEMIOLOGY OF ASEPTIC LOOSENING

The most complete epidemiological data on total joint replacements can be found in the Swedish National Hip Arthroplasty register. This hip register, began in 1979 and to date 169,419 primary total hip replacement procedures have been registered. The Swedish Hip register reported that approximately 10,000 total hip replacements were performed annually (100/100,000 inhabitants), and this number increased continuously up to 1992. There were 13,591 cases of revision total hip replacements between 1979 to 1998. Of those, 11,543 were revised for the first time and 1,713 were revised for the second time. The major reasons for revision were aseptic loosening in 76%, technical error and dislocation in 8.5% and deep infection in 7.5% (2).

The Danish Hip Arthroplasty Register was established in 1995 to monitor the results of total hip replacement in the whole of Denmark with a population of 5.3 millions. 3,343 revision total hip replacements were recorded between 1995 – 1999. The most common indication for revision surgery was aseptic loosening in 63% of the revision cases (6).

In other smaller studies the findings were similar. In 1999, Fender et al using the Trent Regional Arthroplasty register, reported a 5 year outcome of Charnley total hip replacement from a single health

region in England. After 5 years, 35 out of 1080 prostheses (3.2%) had undergone revision. Aseptic loosening was the commonest indication for revision surgery amounting to 40% of the cases (7). Aseptic loosening as the major cause of long term failure of total hip arthroplasty was also reported by Garrelick et al (8), Salvati et al (9), Schulte et al (10) and Wroblewski (11) in four separate studies. These results are summarised in table 2.1.

Most of the epidemiological studies came from the developed countries where total joint replacements are an established treatment for osteoarthritis. However, total joint replacements are being increasingly performed in the developing nations. A retrospective study in Malaysia looked at the outcome of 133 total hip replacements performed between 1987 and 1996. The Malaysian Government National Joint Replacement Center showed the most common cause of failure was aseptic loosening with 9.2% of the primary joint replacements revised at a 5 year average follow-up (12). The results showed that aseptic loosening is not only a problem faced by orthopaedic surgeons in the developed nations but affects the developing nations as well.

As the life expectancy of population age continue to increase, the demand for total joint replacements will increase. There has also been a trend toward inserting these components in younger patients. In 1994, Kavanagh et al showed that the probability of revision

surgery increases inversely with age: 7.5% for those aged between 65 and 70, 13% for those aged between 59 and 65 and an unacceptable 27% for those 59 or younger at the time of surgery (13). These factors ensure that the number of cases of aseptic loosening and revision surgery will continue to increase.

Author	Number	Follow up (year & percentage)	Loose	Revision rate
Garelick et al (8)	95	12 to 16 (100%)	5% cup 7% stem	10.5%
Salvati et al (9)	100	9.5 to 11 (67%)		3%
Schulte et al (10)	322	20+ (98.5%)	9% stem	10%
Wroblewski et al (11)	1324	18 to 26 (15%)		14%

Table 2.1: Summary of a selection of published results of total hip arthroplasty follow-up.

2.2 AETIOLOGY OF ASEPTIC LOOSENING

The aetiology of prosthesis loosening is multifaceted. Despite extensive research, the mechanisms responsible for aseptic loosening are not completely understood. The most widely accepted theory for this is that chronic inflammation induced by biomaterial wear particles leads to osteoclastic activation and periprosthetic bone loss. Harris et al reported the presence of debris particles around loose implants and the bone interface in 1976 (14). Wear particles including metal (15, 16), polyethylene (PE) (17, 18), polymethylmethacrylate (PMMA) (19) and ceramic (20) have been shown to activate marcrophages. The debris activated macrophages fuel the inflammation process by cytokine-mediated pathways and direct bone resorption.

Horowitz et al used the electron microscope to analyse the interface membrane of aseptically loosened joints, and they found that only small debris particles (1 – 12 micrometers) were phagocytised by macrophages. In the same study they also demonstrated that macrophages exposed to small PMMA particles (12 micrometers or smaller) released a high amount of the bone resorbing mediator, tumour necrosing factor (19). Kobayashi et al using a combination of tissue digestion and electron microscopy found that there was no correlation between the presence of osteolysis and the morphology or size of the PE wear particles. However, there was a highly

significant association between the number of particles and the presence of osteolysis. They reported that the threshold for the occurrence of osteolysis was 10 billion particles per gram of wet interface tissue (21). Kadoya et al showed that the number of PE particles was associated with osteolysis but not the size of the particles. They also reported that there was no significant difference in the average particle size (0.82 micrometer) between the well fixed (recurrent dislocation) and loose prosthesis (aseptic loosening) (22). These studies showed that wear debris played an important role in the process of aseptic loosening. Both Kadoya and Kobayashi showed the importance of debris concentration in the process of aseptic loosening and the reported average debris particle size was 0.82 micrometer (21, 22). Though Horrowitz did not test the potency of particles below 1 micrometer in size, he did speculate that any particles below 12 micrometer could activate macrophages. From these studies it is reasonable to conclude that both particle size and concentration are equally important in the process of aseptic loosening (19).

Mechanical factors also play an important role in the process of aseptic loosening. As clinical experience has shown, failure of mechanical integrity of the interface and the subsequent gross movement of the component results in catastrophic bone loss (23, 24). Ferrier et al showed that applying cyclical pressure to

macrophages can lead to increased secretion of inflammatory cytokines (25). Jones et al reported that in an in vivo canine model of aseptic loosening, inflammatory response to debris particles was much slower in stable implant (26).

Evans et al speculated that allergy to the implanted material may cause vasculitis, secondary bone necrosis, and subsequent bone resorption and prosthesis loosening (27). The role of metal allergy and aseptic loosening remains uncertain.

Jones et al suggested a "double hit" hypothesis for the pathogenesis of aseptic loosening where two different stimuli, one biological (wear particles) and the other mechanical (movement), act synergistically towards periprosthetic osteolysis (26).

2.3 BIOLOGY OF ASEPTIC LOOSENING – INTERFACE MEMBRANE

In recent years, attention has been focused on the potential pathological importance of a synovial-like membrane which develops between implant or cement and bone. This membrane is also known as interface membrane.

In this section the role of interface membrane in aseptic loosening will be discussed.

2.4 INTERFACE MEMBRANE – CELLULAR PROFILES

In 1976, a team of surgeons working in Boston, USA presented a series of case reports on extensive localised bone resorption on patients following total hip replacements where no infection or tumor were found. During revision surgery, the prosthesis was found to be only slightly loose. In all the patients, friable yellowish tissues were found between the bone and cement interface. None of these specimens grew any bacterial organisms. When the specimens were examined histologically there were sheets of macrophages, giant cells and multiple small fragments of birefringent material, but no inflammatory cells. The cause of the loosening was unknown at the time and the surgeons speculated that this could be due to tissue response to fragmented methacrylate (14). In 1983 Goldring, from the same institution in Boston, examined the membrane between the

bone-cement interface of twenty patients with aseptic loosening of total hip joint replacements. The membrane was studied histologically, histochemically, by cell culture, by organ culture, and by assessment of its ability to synthesize prostaglandin E_2 and collagenase. Histological findings were similar to those reported by Harris. Histochemical evaluation revealed the presence, within the interface membrane, of cells which stained positively of acid phosphatase and lysozyme, functional enzyme markers that are commonly associated with macrophages. Three distinct types of cell were identified in the cell culture. The first was dendritic, or stellate, like cells similar to those seen in rheumatoid tissue; the second type was marcrophage and the third type was fibroblast. In the organ culture these tissues were found to release large quantities of prostaglandin E_2 and collagenase. It was postulated that this tissue might play a major role in bone resorption and that bone cement might be responsible for the formation of this membrane (28). In the same year, Linder et al reported similar results. In their histological study of specimens from 21 aseptically loosened joints, they reported the presence of a connective tissue layer between the bone and the cement in all the specimens. Fibrocytes, macrophages and foreign body giant cells were found in these specimens. At the bone-interface membrane border, both bone resorption and bone formation were present. The majority of the specimens showed predominantly

bone resorption (29). Saffar and Revell examined the interface membranes of 10 patients with aseptic loosening. They reported the presence of T-lymphocytes in all the specimens (30) and this constituted approximately 5% - 10% of the total cell population in the interface membrane (28, 31, 32, 33). The presence of T-lymphocytes suggests a possible role for immunological processes in aseptic loosening, but this is controversial and there are studies which advocate (33, 34, 35) and refute (36, 37, 38) the role for T cell modulation of macrophage response to biomaterials.

We now know that the inflammatory cells found in the interface membrane have the ability to resorb bone. Athanasou, Quinn and Bulstrode in 1992 showed that both macrophages and macrophage polykaryons from interface membrane of aseptically loosened joints were able to produce resorption pits when cultured with cortical bone slices (39). In 1995, Kadoya et al showed that the macrophages taken from aseptic loosened hip joints contained the necessary enzymes for bone resorption and also cellular microstructures for bone resorption (40, 41). Recently it has also been shown that activated fibrocytes from aseptic loosened joints can activate macrophages to resorb bone (42).

2.5 INTERFACE MEMBRANE – CYTOKINE PROFILES

The term 'cytokine' is applied to proteins produced by cells in response to a variety of inducing stimuli; they are secreted by their producer cells and then influence the behaviour of target cells. Cytokines are produced by interface tissue, but their relative importance in the resorption of periprosthetic bone is controversial. This is a brief overview of the biochemical profiles of interface membrane.

Prostaglandin:

Prostaglandin E_2 (PGE_2) has long been known to be a potent stimulator of bone resorption. Some of the effects of the cytokines on osteoclastic bone resorption in vivo as well as in vitro are mediated through prostaglandin synthesis (43, 44).

Early studies by Goldring et al showed the presence of PGE_2 and collagenase in the interface membrane of aseptically loosened hip joints. The presence of PGE_2 in interface membrane led Goldring et al to postulate that interface membrane has the capability to resorb bone (28).

Interleukins:

The generic name "interleukin" was proposed in 1979 for factors produced and released by activated lymphocytes that act on other lymphocytes to produce biological effects. We now know that

interleukins are also produced by cells of other hematopoietic lineages, such as monocytes (45).

Interleukin-1 (IL-1) is a powerful stimulator of osteoclastic bone resorption (46, 47). IL-1 stimulates the formation of osteoclasts from progenitor cells (48). IL-1 also activates mature osteoclasts to resorb bone (49). Saffar and Revell in 1994 sampled the interface membrane from 10 patients with aseptic loosening during revision surgery. Seven of these patients had aseptically loosened hip arthroplasties and 3 had aseptically loosened knee arhtroplasties. Bone cement was used in 5 of the 10 cases. By using immunohistochemical-staining method, they demonstrated the presence of IL-1 in the interface membrane, and this was found mainly in the macrophages (50).

In another study, Kim et al investigated the IL-1 activity in the interface membrane of aseptically loosened uncemented total hip arthroplasty, and they found IL-1 activity was significantly higher in implants containing polyethylene, titanium alloy and cobalt chrome alloy. There was also a strong correlation between IL-1 activity level and radiographic osteolysis (51).

Interleukin-6 (IL-6) is synthesized by T-lymphocytes, monocytes, macrophages, fibroblasts and endothelial cells, making it, together with IL-1, one of the most ubiquitously active cytokines (52,53). It is known to stimulate osteoclastic bone resorption (49, 52, 53, 54).

Goodman et al reported that IL-6 was present in the interface membrane. Both IL-1 and IL-6 positive cells were present in greater numbers in specimens taken from loose cemented implants compared with well fixed cemented implant (33).

Interleukin-8 (IL-8) is produced by monocytes, macrophages and fibroblasts from various tissues (55, 56). In osteoclasts, IL-8 can stimulate the motility and increases the spread area of osteoclasts. It is speculated that the main function of IL-8 in bone remodelling is to cause osteoclasts migration from one resorption site to another (57). In 1995, Sabokbar and Rushton determined the concentration of PGE_2, IL-6 and IL-8 in the hip synovial fluid of 20 patients undergoing primary and revision total hip arthroplasties. Using a quantitative immunoassay kit, they found IL-8 was increased in the revision group (58). Lassus et al, using immunohistochemical staining, showed an increase of IL-8 producing cells in the interface membrane of aseptically loosened hip compared with normal knee synovial membrane (59).

Human interleukin-11 (IL-11) has a broad spectrum of activity similar to that of IL-6. It is able to induce the formation of osteoclasts and bone resorption (60, 61). Xu et al analysed tissue specimens taken from interface membrane of aseptically loosened joints and synovial membranes of osteoarthritic hip joints using immunohistochemical method. They found an increase of IL-11

positive cells in the interface membrane compared to the synovial tissue of osteoarthritic hip (62).

Which of these interleukins is the most important in the process of aseptic loosening is at present unknown.

Tumour Necrosis Factor Alpha (TNF-α):

As with many cytokines, "tumour necrosis factors" (TNFs) reflect the history of these agents, and are misleading because, although TNFs can induce the regression of tumours, it gives no clue to their many other actions. TNF alpha (TNF- α) is produced mainly by macrophages in response to activation by agents such as bacterial endotoxin and wear debris. Other cell types can also produce TNF- α such as T cells and fibroblasts. TNF- α plays an important role in control of inflammatory response. It has the abilily to induce differentiation and production of cytokines in the monocyte/macrophage lineage (63). TNF- α has biological effects similar to IL-1. It has been shown to stimulate bone resorption (64).

In 1993, Jiranek et al reported the presence of TNF- α in the interface membrane of loosened implant (65). Goodman et al also reported large numbers of cells staining positive for TNF- α in the interface membranes of both aseptically loosened cemented and cementless implants (33). Jones et al showed TNF- α was mainly present in the macrophages and fibroblasts of interface membrane (32).

RANK Ligand (RANKL):

RANKL is also known as tumour necrosis factor-related, activation-induced cytokine (TRANCE). It has been shown to stimulate osteoclastic bone resorption by acting directly on cells in the osteoclast lineage (66).

Recent study by Haynes et al showed that interface membranes tissue samples showed an increase expression of mRNA corresponding to RANKL (67). Itonaga et al further showed that RANKL can transform macrophages isolated from interface membrane of aseptically loosened prosthesis into bone resorbing cells (68).

Macrophage Colony Stimulating Factor (M-CSF):

Monocytes and macrophages are the main sources of M-CSF in normal tissues. M-CSF has been shown to stimulate macrophages to secrete IL-1 and PGE_2. It also enhanced phagocytosis and cell killing (70).

Haynes et al showed an increase expression of mRNA corresponding to M-CSF in the interface membrane of aseptically loosened joints (67).

Epidermal Growth Factor and Transforming Growth Factor Alpha:

Stern et al and Tashjian et al showed that epidermal growth factor (EGF) and transforming growth factor alpha (TGF α) were local regulators of bone metabolism and stimulate bone resorption (71, 72).

In 2000, Xu et al showed that both EGF and TGF α were produced at the interface membrane retrieved from aseptically loosened joint (73).

Platelet Derived Growth Factor:

Platelet derived growth factor (PDGF) is a mitogen for connective tissue cells. It plays an important role in both inflammations and wound healing (74). PDGF was originally isolated from platelets, but is now known to be produced by other cell types including osteoblasts, osteoclasts, macrophages, endothelial cells and fibroblasts. PDGF has been shown to increase bone resorption (75). Xu et al showed that the production of PDGF was increased in the interface membrane of aseptically loosened joint. The PDGF chains in the interface membrane were mainly found in macrophages, fibroblasts and endothelial cells (76). The roles of EGF, TGF α and PDGF in aseptic loosening remain unknown.

2.6 MECHANISMS OF CELLULAR RECRUITMENT IN ASEPTIC LOOSENING

Cytokines and cellular interactions play a major role in the process of aseptic loosening. Previous study by Pollice et al showed that exposure of macrophages to polymethylmethacrylate (PMMA) particles leads to the production of mediators that can stimulate the recruitments of osteoclasts (77). It has also been shown by Horrowitz et al that the recruitment of osteoclasts is dependent upon the presence of osteoblasts (78). The release of mediators by macrophages following PMMA or polyethylene (PE) exposure may stimulate osteoblasts to produce mediators that ultimately lead to the recruitment of cells into the area of the bone cement interface. This hypothesis is supported by studies demonstrating that macrophages exposed to PMMA or PE particles produced mediators that stimulate osteoblasts to release arachidonic acid products and PGE_2 (78, 79, 80).

PMMA particle exposure has been demonstrated to stimulate TNF production by macrophages (19). M-CSF is a cytokine released by osteoblasts in response to TNF and is known to stimulate the recruitment of macrophages into sites of inflammation (81). IL-6 is also released by osteoblasts in response to TNF and is known to stimulate the recruitment of osteoclasts into sites of inflammation (82). Horrowitz and Purdon demonstrated that the mechanisms of

cellular recruitment in aseptic loosening were initiated by TNF production by macrophages following PMMA particle exposure. TNF stimulate osteoblasts to produce M-CSF, IL-6 and PGE_2, which leads to recruitment of macrophages and osteoclasts into the area of bone-cement interface (78) [Fig. 2.1].

Currently there are two hypotheses concerning which cell type is responsible for osteolysis in aseptic loosening. The first hypothesis suggested that increased bone resorption in aseptic loosening was due to macrophages. This hypothesis was based on the report that there were approximately 25-fold more macrophages on bone surfaces surrounding loose implants compared with bone surrounding well fixed implants (83). The alternative hypothesis suggested that increased recruitment of osteoclastic precursor cells and their differentiation into mature osteoclasts was responsible for the increase of bone resorption in aseptic loosening. This hypothesis was based on a study which showed that wear particles increased recruitment of precursors and differentiation of osteoclasts (84).

It is possible that both mechanisms may contribute to the process of aseptic loosening. It remains uncertain as to which of the above mechanisms predominate in the process of aseptic loosening. Further research is needed in this area.

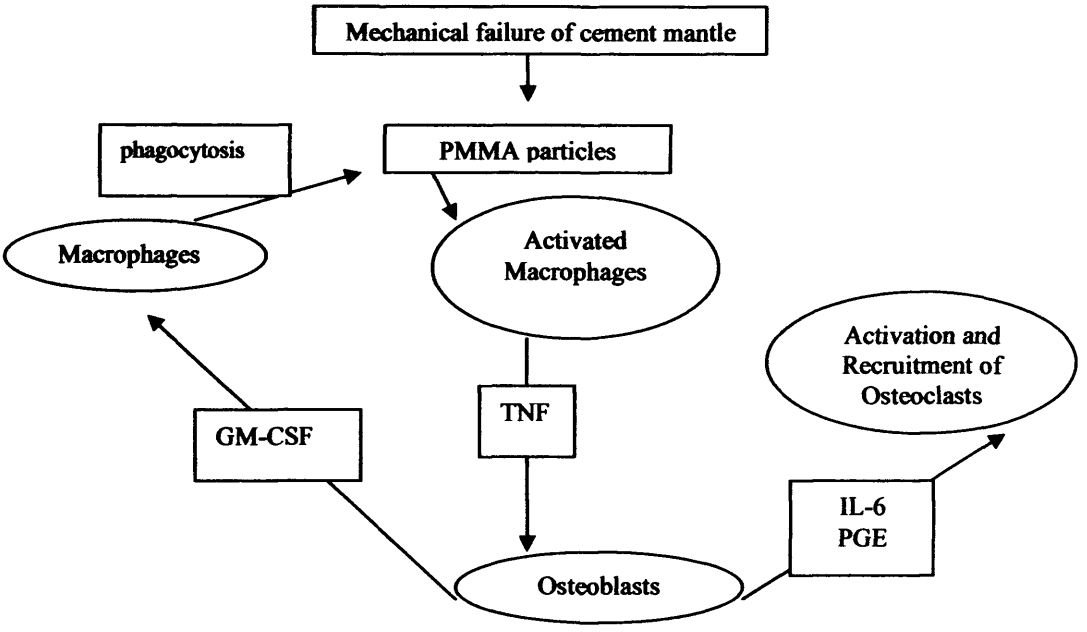


Fig 2.1: The process of cellular recruitment in aseptic loosening is initiated when the mechanical failure of the cement mantle leads to the production of PMMA particles. These particles are phagocytized by macrophages leading to the production of TNF. TNF stimulates surrounding osteoblasts to produce GM-CSF, IL-6 and PGE-2 which leads to recruitment of more macrophages, osteoclasts and perhaps other inflammatory cells into the area of the bone-cement interface. The recruitment of these cells potentiates this process leading to bone resorption and ultimately, clinical loosening (78).

2.7 MATRIX METALLOPROTEINASES (MMPs) AND BONE RESORPTION

In order to understand the process of bone resorption in aseptic loosening first we have to understand the normal process of osteoclastic bone resorption. The following is our current knowledge of normal bone resorption and the role of MMPs in this process.

The human MMPs are a family of at least 16 homologous zinc dependant enzymes, which were able to degrade almost all components of extracellular matrix (85). These enzymes played a major role in normal and pathological tissue remodelling (86).

The osteoclast is a giant multinucleated cell, up to 100 micrometers in diameter, containing four to twenty nuclei (87). Osteoclasts resorb bone by acidification and dissolution of hydroxyapatite crystals and proteolysis of bone matrix within the sealing zone. The first process during osteoclastic bone resorption is mobilisation of hydroxyapatite crystals by digestion of their link to collagen via the noncollagenous proteins, and the low pH dissolves the hydroxyapatite crystals, exposing the bone matrix. This is followed by digestion of the remaining collagen fibres by enzymes secreted by the osteoclasts. The enzymatic mechanisms by which the organic matrix of bones is removed are still incompletely understood. The evidence to support the role of cathepsins and MMP in bone resorption is circumstantial (88).

It has been shown that lysosomal cycteine proteinases including cathepsins L, B and N were involved in osteoclastic bone resorption (89, 90, 91, 92). These proteinases can effciently degrade type 1 collagen mainly found in bone at acid pH (93). Vaes reported that Cathepsins L and B were present in homogenates of bone tissues (94). In addition specific inhibitors of cysteine proteinases were shown to reversibly inhibit bone resorption both in vitro and in vivo (92, 95).

Another group of proteinases related to bone resorption were members of the MMPs. Vaes reported that MMP-1 (interstitial collagenase) was present in the culture of bone explants (96), and Eeckhout et al reported similar findings in extracted mineralised bone (97). Immunolocalization studies demonstrated localization of MMP-1 in osteoblasts (98) and in rodent osteoclasts and the subosteoclastic bone resorbing compartment (99). Involvement of MMPs in bone resorption was further supported by a study showing that specific synthetic inhibitors of MMPs inhibit the parathyroid hormone-induced bone resorption of explanted mouse calvaria by preventing collagen degradation (100). Case et al showed that MMP-3 was immunolocalized within osteoclasts in the rat experimental arthritis model (101). Okada reported that MMP-9 was produced by human osteoclasts, and he also showed that MMP-9 could degrade type 1 collagen in demineralised human bone. MMP-9 remained

active in sub-osteoclastic environment i.e. the presence of acidic (pH 2.3) and high calcium (100mM) environment (102).

These studies showed that both MMPs and cathepsins were involved in bone resorption. It is not possible at present to conclude which of these enzymes are pre-dominantly responsible.

2.8 MATRIX METALLOPROTEINASES (MMPs) AND ASEPTIC LOOSENING

Takagi et al using zymographic and densitometric analysis revealed elevated production of MMP-2 and MMP-9 in tissue extracts from interface membrane of aseptically loosened implants when compared with non inflammed knee synovial tissue acting as control (103). Yokohama et al, using an immunohistochemical staining method, showed that in the interface membrane, MMP-9 was strongly positive in multinucleated giant cells and MMP-2 were mainly found in fibroblasts. There was also an increase of MMP-9 mRNA expression in the cells detected using in situ hybridization technique (104). In 2000 Takei et al showed that the mRNA expression for MMP-1, MMP-3, MMP-9, MMP-10, MMP-12 and MMP-13 was significantly higher in the interface membrane of aseptically loosened joint compared with synovium taken from osteoarthritic joint (105).

MMPs may play a dual role in aseptic loosening. The first role involved active tissue remodelling within the periprosthetic

connective tissue bed and this combined with cyclical loading, may lead to loosening of the prosthesis (26, 103). Secondly MMPs had been shown in previous studies to be actively involved in bone resorption (102). The presence of high concentration of MMPs in the macrophages found in the interface membrane suggest MMPs may be directly involved in periprosthetic bone resorption (103, 104, 105). MMPs may represent the final common pathway in aseptic loosening irrespective of the aetiology. MMPs inhibition can potentially stop the process of aseptic loosening. A large part of this thesis concentrates on exploring the possibilities of using currently available pharmacological agents to inhibit the MMPs pathway in aseptic loosening.

2.9 SUMMARY OF THE LITERATURE REVIEW

1. Aseptic loosening is the most common mode of long term failure in total joint replacements. The incidence of aseptic loosening is bound to increase in the future.
2. Two factors were indentified as the possible initiators of aseptic loosening. These are biological factors (wear particles) and mechanical factors (movement) acting synergistically on periprosthetic osteolysis.
3. Both biological and mechanical factors initiate a biological host response. They activate marcophages and this is followed by the released of various cytokines such as TNF α, IL-1, IL-6, IL-8, IL-11, PGE_2, M-CSF, RANKL, EGF, TGF α and PDGF. These cytokines have been shown to stimulate bone resorption, but the relative importance of these biochemical agents in aseptic loosening remains unknown.
4. These cytokines, in turn, recruit and activate more cells (marcophages, fibrocytes, foreign body giant cells and T lymphocytes). This lead to the formation of interface membrane between the bone and prosthesis.
5. The activated cells in the interface membrane have the ability to resorb bone directly and indirectly stimulate

resident osteoclasts to resorb bone. The net result is periprothetic bone loss and aseptic loosening.

6. MMPs are enzymes that have the ability to degrade extracellular matrix and are believed to play a major role in normal and pathological bone resorption. These enzymes are found in osteoclasts and macrophages of interface membrane. It may represent the final common pathway in periprosthetic osteolysis.

7. Inhibition of MMPs may be the key to stopping aseptic loosening.

CHAPTER 3

CURRENT KNOWLEDGE OF PHARMACOLOGICAL TREATMENT OF ASEPTIC LOOSENING

3.1 SURGICAL TREATMENT OF ASEPTIC LOOSENING

At present revision surgery is the only option for the treatment of aseptic loosening. Unfortunately this type of surgery carried with it a high morbidity rate. Kavanagh et al from the Mayo clinic reported the results of 166 revision total hip replacements for aseptic loosening. The average follow up period was 4.5 years. Complications that occurred with revision surgery included deep sepsis, superficial wound infection, dislocation, intraoperative femoral fracture and postoperative femoral fracture. These complications were higher compared to primary total hip replacements. They also reported probable loosening in 21% of the revised acetabular components and 44% of the revised femoral components (106).

Pellici et al reported the long-term follow up results of 99 patients who had undergone revision surgery for aseptic loosening of total hip arthroplasty. The average follow up period was 8.1 years. They found almost a third of these revised arthroplasties failed within this period (107). Stromberg & Herberts in 1994 reported the results of 10 year follow up following revision total hip replacements. They found 83% of these revision arthroplasties had undergone a second revision surgery (108).

These studies showed that revision arthroplasty surgery generally carried a higher complication rate compared to primary surgery. The majority of the revised joint replacements failed within 10 years of revision. Unfortunately presently this is the only treatment option available to patients with aseptic loosening despite the poor long-term results.

3.2 PHARMACOLOGICAL TREATMENT OF ASEPTIC LOOSENING

Our understandings of aseptic loosening at cellular level has improved over the years and by inhibiting specific cellular pathway using pharmacological agents, may prevent or halt the process of aseptic loosening. The idea of treating aseptically loosened hip with pharmacological agents is not new and there had been various studies in the past showing promising results.

In 1996, Pandey et al showed that bisphosphonate, disodium ethane-1, 1-diphosphonate (EHDP) when cultured with PMMA activated macrophages significantly reduced bone pit formation on cortical bone slices (109). In the same year Horrowitz et al using radioactive calcium 45 murine calvaria model also showed that bisphosphonates, disodium pramidonate has the ability to inhibit bone resorption by PMMA activated macrophages (110).

In 1997, Shanbhag et al using in vivo canine model of aseptic loosening showed that oral bisphosphonates, Alendronate could prevent periprosthetic bone loss but not the foreign body reaction associated with aseptic loosening (111).

The exact mechanism of action of bisphosphonates is not fully understood. Previous studies have shown that bisphosphonates act as synthetic analogs of pyrophosphate that bound to hydroxyapatite found in bone. The bisphosphonates accumulated within the bone

became liberated in acidic environment within the clear zone beneath the osteoclasts ruffled border (112). The free bisphosphonates increased the membrane permeability of the ruffled border and disabled the osteoclasts, preventing bone resorption (112, 113).

Another potential mechanism for preventing aseptic loosening of joint arthroplasties is pharmacological inhibition of activated macrophages. Tumour necrosis factor alpha (TNF - α) is believed to be one of the most important mediators in the development of particle induced osteolysis and is one of the earliest proinflammatory mediator released (114, 115).

In 2000, Schwarz et al demonstrated that tumour necrosis factor alpha (TNF - α) inhibitor, Pentoxifylline was as effective as alendronate in preventing debris induced osteolysis in an in vivo murine model (115). In 2001, Pollice et al showed that when pentoxifylline were administered orally to human volunteers, their peripheral blood monocytes showed a significant reduction in TNF - α released when exposed to titanium particles compared with monocytes taken from the same individuals before taking the pentoxifylline (116).

These studies showed that the process of aseptic loosening could be halted in the laboratory using presently available pharmacological agents by blocking the process at various pathways. Part of this

thesis is to investigate the potential of currently available drugs such as doxycycline and statins against the process of aseptic loosening.

3.3 DOXYCYCLINE AND ASEPTIC LOOSENING

Doxycycline is a semi-synthetic tetracycline antibiotic that became available for clinical use in 1966. Like other tetracyclines it has a wide spectrum of activity. In addition to its antibiotic activity it also a chelating agent and will chelate Ca^{2+}, Mg^{2+} and Al^{3+} ions.

Antimicrobial action

Doxycycline is primarily a bacteriostatic antibiotic and its main mechanism of action is on protein synthesis. Doxycycline passes directly through the lipid bilayer of the bacterial cell wall. In addition, an energy-dependant active transport system pumps the drug through the inner cytoplasmic membrane. Once inside the bacterial cell, doxycycline inhibits protein systhesis by binding specifically to the 30S ribosomes. The drug appears to prevent access of aminoacyl tRNA to the acceptor site of the mRN-ribosome complex. This prevents the addition of amino acids to the growing peptide chain. Doxycycline will impair protein synthesis in mammalian cells if used at very high concentrations. However, these cells lack the active transport system found in bacteria (117).

Non antimicrobial action

In 1982, Professor Golub was studying the role of collagenase in peridontal disease. Their model system was the diabetic rat, rendered so by injection of streptozocin. Such animals develop advanced gingivitis with concomitant alveolar bone loss and therefore serve as a good model for gingival disease. It had been known for sometime that these animals developed pathologically excessive levels of collagenase in skin and gingiva, with concomitant loss of collagen content. In a study designed to elucidate the role of microorganisms in the induction of excessive collagenase, Golub and his co workers decided to eliminate the bacterial factor by treating the animals with antibiotic. They chose a tetracycline, since drugs in this class were known to be effective adjuncts to therapy in human gingival disease. In order to ensure scientific purity, they decided to run parallel experiment in germ-free animals, thus testing the effect of the drug in a situation where its bacteriostatic properties would presumably be irrelevant. When the tissues were harvested and analysed for levels of collagenase activity, they were surprised to note that the tetracyclines had a profound effect in lowering the enzymatic activity in both the germ-free and the conventional animals (118).

From this seminal observation, extensive follow-up studies have shown that tetracyclines, especially semisynthetic agents such as minocycline and doxycycline, are excellent inhibitors of collagenase

both in vitro and in vivo (119, 120). Based on studies with purified collagenase from rabbit cornea, the inhibitory activity concentration of tetracycline that inhibited 50% of enzyme (MMP) activity (IC_{50}) for doxycycline, minocycline, and tetracycline was 15, 190, and 350 microM, respectively. It has been proposed that the greater effect of doxycycline was related to its stronger binding of zinc cation at the active site of the enzyme (121).

The effects of tetracyclines on bone were studied soon after the discovery of their anti- collagenase properties (122). The roles of MMPs in bone resorption and aseptic loosening were discussed in previous chapter. However, apart from their anticollagenase property, tetracyclines are also potent inhibitors of osteoclasts function and, therefore bone resorption. Tetracyclines have been shown to inhibit osteoclasts function by

1) altering the responsiveness of osteoclasts to elevated extracellular calcium (123).
2) decreasing the ruffled border area of the osteoclast (124)
3) diminishing acid production (125)
4) inducing osteoclast cell retraction (123).

In 1987, Greenwald et al showed that tetracyclines can inhibit extracellular collagenase activity in human synovial tissue and fluid from rheumatoid arthritis (126). In 1990, Golub et al observed that

doxycycline can prevent the development of osteopenia in the long bone of diabetic rats (127).

All the studies so far suggest that tetracyclines could influence bone metabolism especially bone resoprtion by various pathways. Studies on aseptic loosening showed that MMPs may play a major role in the process of aseptic loosening and these enzymes are present both in the osteoclasts and macrophages, which are largely believed to be the cells responsible for bone resorption. The effect of tetracyclines on aseptic loosening as far as we know has never been investigated. Part of this thesis is to investigate the effect of doxycycline on the process of aseptic loosening. Doxycycline was chosen in this project because it has been shown previously to be the most potent MMP inhibitor (119).

3.4 FLUVASTATIN AND ASEPTIC LOOSENING

Statins (HMG-CoA reductase inhibitors) have been used for the treatment of hypercholesterolemia and coronary heart disease for more than a decade. Their efficacy and safety have been well documented. Their mechanism of action is the inhibition of the rate-limiting enzyme of cholesterol synthesis, namely 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (128). Fluvastatin is the first wholly synthetic HMG-CoA reductase inhibitor to become available. Fluvastatin sodium is prepared by chemical synthesis and is not fungus derived, as is the case for other statins. Fluvastatin sodium is a mevalonolactone derivative of a fluorophenyl-substituted indole ring. The substituted indole portion of the molecule mimics mevalonate, thus reversibly inhibiting the enzyme. Unlike simvastatin and lovastatin, fluvastatin is not a prodrug and is provided in its active hydroxy form (129).

HMG-CoA reductase is one of the major enzymes in the mammalian cell mevalonate pathway which yields a series of isoprenoids that are vital for diverse cellular functions such as cellular signalling, cellular differentiation and proliferation, myelination, cytoskeleton dynamics, and endocytotic/exocytotic transport (130) (Fig. 3.1). Hence inhibition of HMG-CoA reductase by statins has the potential to result in pleiotropic effects.

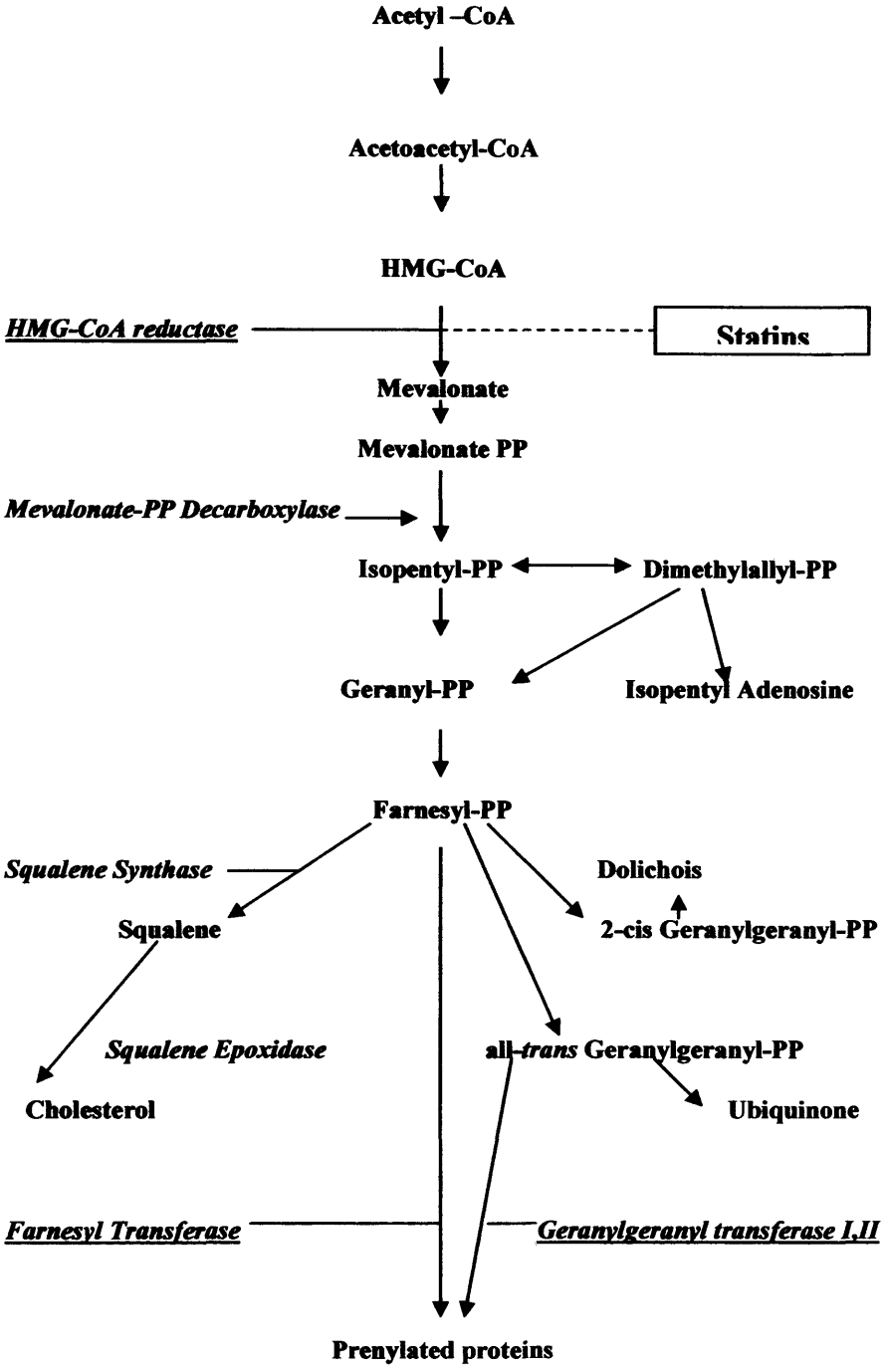


Fig 3.1: The mammalian cell mavalonate pathway.

PP = pyrophosphate. Corsini et al 1999 (130).

Statins had also been shown to reduce the secretion of matrix metalloprotenaises (MMPs) by macrophages (131, 132). Recently it has been shown that fluvastatin, at a concentration as low as 1microM, inhibits the activity of gelatinase-B (MMP-9) in human monocyte-derived macrophages (133). In addition, fluvastatin can inhibit MMP-9 secretion in a concentration-dependent manner; this inhibitory effect could be prevented by the simultaneous addition of mevalonate (133).

In 1999, Mundy et al showed that incubation of cultured mouse or human bone cells with statins enhanced the expression of bone morphogenetic protein-2 (BMP-2) mRNA (134). Mundy and co workers further showed that statins added to neonatal mouse calvaria bone in organ culture increased new bone formation by approximately 2 – 3 times (134). Further in vivo study in mice confirmed that a 50% increase in new bone formation was detected in mice that were given subcutaneous statins injections (134). Similarly, 35-day oral administration of statins to both intact and ovariectomised rats resulted in increased of 39% - 94% in trabecular bone volume (134). These anabolic effects were associated with a decrease in osteoclast numbers (134).

In 2001, Maeda et al demonstrated that alkaline phosphatase activity and bone mineralization were enhanced when non-transformed osteoblastic cells and rat bone marrow cells were incubated with

statin. These results showed that statin has anabolic effects on bone through the promotion of osteoblastic differentiation (135).

Previous studies showed that wear debris from joint arthroplasties can transformed macrophages into bone resorbing cells and MMPs levels were raised in the interface membrane of aseptically loosened joints (39, 103). MMPs are believed to play an important role in the osteolytic process of aseptic loosening (102, 103). In previous study on the prevention of artherosclerotic plaque, Fluvastatin was found to have the ability to inhibit MMPs activities especially MMP-9 in human monocytes derived macrophages (133). The MMPs inhibition property of fluvastatin together with the anabolic effect on bone could be exploited for the use in the prevention and treatment of aseptic loosening. To date no one has investigated the potential pharmacological effect of fluvastatin in aseptic loosening. Part of this thesis is to find out if fluvastatin has the ability to inhibit bone resorption by interface membrane cells taken from aseptically loosened hip replacements.

3.5 MONITORING PHARMACOLOGICAL TREATMENT OF ASEPTIC LOOSENING

To best apply pharmacological therapy for osteolysis in aseptic loosening a reliable, objective and non invasive clinical tool is required to diagnose and assess treatment of aseptic loosening. Radiographic changes are slow in onset and become evident only after extensive resorption has occurred. Dual-energy x-ray absorptiometry is the gold standard in assessing bone mass but it is limited by being a static measurement that does not offer any information on the current state of bone turnover (136). A biochemical marker would be ideal for diagnosing and assessing treatment noninvasively prior to any radiographic evidence of significant bone destruction. In addition, with evidence of such destruction, a biologic marker would be essential in monitoring whether the osteolysis was progressing or being controlled.

Biochemical assays for monitoring bone resorption usually rely on target enzymes synthesized by osteoclasts or metabolites of collagen generated by osteoclasts during bone resorption. This section gives a brief overview of the currently available bone resorption marker and this is followed by its application in aseptic loosening in the next section.

Hydroxyproline

Hydroxyproline is the traditional bone resorption marker but its usefulness as a clinical marker is blunted by contribution from diet, lack of specificity to bone collagen, degradative losses of the free amino acid in the liver, and the relatively tedious chemical assays used for its determination (137).

Hydroxylisine Glycosides

All collagenous proteins contain glycosylated hydroxylysine residues. There are two forms, glucosylgalactosylhydroxylysine (GGHyl) and galactosylhydroxylysine (GHyl). They appear to be excreted quantitatively in urine as free hydroxylysine glycosides, presumably as products of collagen catabolism (138, 139). Human bone collagen is unusual in containing more Ghyl than GGHyl (in contrast to that in skin for example). GHyl also dominates in urine, indicating that bone is the primary source and that GHyl can provide a reasonable index of bone resorption rate. From a clinical perspective, however, the current high-performance liquid chromatography (HPLC) assays are not convenient. If a reliable immunoassay for GHyl be developed, it might have clinical potential (140).

Tartrate-Resistant Acid Phosphatase

Tartrate-resistance acid phosphatase (TRAP) is a prominent product of osteoclasts thought to be active in bone matrix degradation (141). TRAP activity is elevated in serum in clinical conditions that involved bone resorption (142). However, certain other cell types can produce TRAP (143).

Collagen Cross-Links

The collagen cross-linking amino acids, pyridinoline and deoxypyridinoline, have been applied extensively in research as bone resorption markers (144, 145). These complex amino acids form in the fibrils and have no means of metabolism when collagen is degraded. They are excreted in urine where they can be quantified by HPLC relying on their natural fluorescence for detection (146). Approximately 67% are excreted in the form of small peptides and the remainder as free amino acids. Two forms exists: hydroxylysylpyridinoline (or simply pyridinoline, HP, or Pyr) and lysylpyridinoline (deoxypyridinoline, LP, or Dpy). They are posttranslational variants that results from incomplete hydroxylation of lysine residues, at sites that will become cross-links, during the synthesis of collagen types I, II, III and IX (147).

Pyridinolines form mature cross-links in collagens of most connective tissues other than skin. Because bone is a major reservoir of type I collagen in the body and is believed to remodel much faster

than most major connective tissues, bone is likely to be the major source of the pyridinoline pool in urine. This conclusion is supported by the similar ratios of Pyr to Dpy in human bone (3.5:1) and urine (range 2:1 – 7:1) (148). Bone is distinguished by its high proportion of Dpy compared with that in most non bone tissues, in which Dpy is usually less than 10% of the Pyr content (147, 148). Urinary Dpy therefore is more specific than Pyr as a marker of bone degradation (149). However, Dpy is not unique to bone and the overall Pyr content of bone collagen is actually quite low (0.3 mole/mole) compared to that in other connective tissues (1.5 mole/mole in cartilage, 0.5-1 mole/mole in vascular tissue, tendon, ligament, fascia, lung, intestine, liver, muscle and so forth (147, 150, 151). Because little is known about collagen degradation rates in tissues other than bone, Pyr and Dpyr results should be interpreted cautiously regarding their specificity to bone metabolism.

Collagen Telopeptide Assays

There are two intermolecular sites of Pyr cross-linking in Type I collagen, N-telopeptide-to-helix and C-telopeptide-to-helix. Two pools of Pyr-containing peptides originating from these sites can be identified in urine. They appear to be discrete, core amino acid sequences attached to the cross-link that resists proteolysis (152). The cross-linked N-telopeptide fraction from urine had a Pyr-Dpy ratio of 2:1, consistent with an origin in bone. Similar domains

prepared from human bone collagen showed that 67% of the Dpy in collagen is located at the N-telopeptide site and 33% at the C-telopeptide site (152, 153). The alpha 2(I) N-telopeptide also appeared to be a favored site of cross-linking in bone collagen (152). Cross-linked N-telopeptides (NTx) have been targeted, therefore, as a promising marker of bone resorption in urine. A monoclonal antibody, mAb 1H11, provides the basis of the NTx immunoassay (154). Osteoclasts generate immunoreactive NTx, but not free pyridinolines (Pyr or Dpy), when cultured on human bone particles in vitro (155). Commercial enzyme-linked immunosorbent assay (ELISA) kit has been developed for both urine and serum (156, 157). Two different assays for collagen Type I cross-linked C-telopeptides are also in use. The I-C-Telopeptide (ICTP) assay is based on a polyclonal anti serum raised against the full-length C-telopeptide domain isolated from bone collagen as a cross linked peptide. It recognizes antigen in serum but not in urine (158). The Cross-Lap assay uses a polyclonal antiserum raised against a synthetic peptide, matching a short segment of the collagen α 1(I) C-telopeptide containing the cross-linking lysine residue (159, 160). This sequence was selected with the expectation that it would be protected from degradation when embodied in Pyr cross-linked structures and excreted in urine (159). CrossLaps is a urine assay, therefore, for cross-linked peptides (CTx) analogous to NTx. ICTP is a serum

assay. From the published clinical data, ICTP and CrossLaps are clearly measuring the products of different pathways (161). The ICTP assay is apparently not an index of normal bone resorption and shows no response in patients on antiresorptive therapies (162). In this study we have chosen to investigate the potential of Dpy and NTx as markers of aseptic loosening based on the findings of previous studies as mentioned above.

Bone resorption markers and aseptic loosening

The idea of using bone metabolites in the diagnosis of aseptic loosening is not new. Previous clinical study showed that urinary NTx and Dpyd were significantly raised in patients with end stage aseptic loosening of total hip arthroplasty. They also found that NTx was the most sensitive and specific compared with Dpyd (163). These findings were further confirmed by a study performed by Antoniou et al, they looked at the urinary NTx levels in four study groups. The first group consisted of patients with aseptic loosening, the second group consisted of patients with total joint replacements without aseptic loosening, the third group consisted of age matched control patients and the fourth group consisted of patients with aseptic loosening treated with alendronate (Fosamax). They found the urinary NTx level was significantly higher in the aseptic loosening group and the urinary NTx level dropped in the group treated with alendronate (164). These clinical studies showed that

NTx could be a potential clinical marker for the diagnosis and monitoring of treatment in aseptic loosening. Previous study by Apone et al showed that NTx was generated directly by osteoclasts during bone resorption but not free pyridinolines in an in vitro model (165). These results imply that the free pyridinolines are not produced directly by osteoclasts during bone resorption in vivo. Degradation to free pyridinolines detected in the urine probably requires further proteolysis in the liver or perhaps the kidneys. This means any condition, which affects the functions of these organs will affect the level of free pyridinolines in the urine (140).

So far NTx appear to be a promising marker for the diagnosis and monitoring of aseptic loosening. Presently the process of osteolysis in aseptic loosening is not fully understood. The osteoclastic cells involved may be either osteoclasts or macrophages or both. Therefore it will be useful to confirm that cells from the interface membrane of aseptically loosened total joint replacements have the abilities to produce NTx during bone resorption. Part of this study is to find out if NTx is produced directly by interface membrane cells of aseptically loosened joints during bone resorption.

3.6 LABORATORY MODEL TO INVESTIGATE POTENTIAL PHARMACOLOGICAL AGENTS OF ASEPTIC LOOSENING

Recent studies have shown that the process of aseptic loosening could be influenced by a numbers of pharmacological agents (109, 110, 111, 115, 116). There are many more pharmacological agents that can potentially influence the process of aseptic loosening waiting to be discovered. An in vitro model of aseptic loosening should initially screen these potential pharmacological agents. An optimal model to test these pharmacological agents should allow rapid multiple testing and be cheap and simple.

The present in vitro models of aseptic loosening either use radiolabelled bone (110, 166) or bone resorption pits (39, 109) to quantify osteolysis. The former model requires the use of radioactive Isotopes and the latter requires very thin bone slices, which are technically difficult to produce and are only semiquantitative.

The previously described biochemical marker such as cross linked N- telopeptide (NTx) may be useful as an in vitro marker. Part of this study was to develop a new in vitro model of aseptic loosening using human cells taken from interface membrane of aseptically loosened joints, non radioactive human bone and NTx as an in vitro marker of osteolysis.

CHAPTER 4

MATERIALS AND METHODS

This chapter constitutes an appendix of definitions which will be referred to in subsequent chapters.

4.1 INTERFACE MEMBRANE

Interface membranes were taken from patients undergoing revision total hip replacement surgery for aseptic loosening. All patients were consented prior to surgery for tissue donation. Once the interface membrane was extracted form the patient, two small samples were sent to the microbiology and pathology departments for culture and histological examination to exclude septic loosening. The remaining tissue was transported in a sterile universal specimen pot containing culture medium to the laboratory. All the tissues were processed within 1 hour after explantation.

In the laboratory, the tissue was processed in a class II culture cabinet with a separate air environment to prevent cross contamination between researcher and specimen.

4.2 ISOLATION OF CELLS FROM INTERFACE MEMBRANE FOR CELL CULTURE

This section outlines the steps used to isolate the cells from the interface membrane for cell culture:

1) The interface membrane was washed thoroughly with warm (37°C) phosphate buffer saline (PBS) to remove all the blood clots. This process was repeated between 5 to 6 times depending on the amount of blood clots present.

2) The interface membrane was cut into 1mm cubed pieces with sterile scalpels and scissors.

3) The cut interface membrane was digested in type I collagenase (Sigma, UK) for 30 minutes in alpha MEM (Gibco BRL, Paisley, UK) culture medium at 37 °C. The concentration of type I collagenase used in this experiment was 1mg/ml.

4) After the first 30 minutes of incubation, 5% trypsin (Sigma, UK) was added to the type I collagenase & tissue mixture. This was incubated further for 1 hour at 37 °C.

5) The partially digested interface membrane was filtered using a 70 micron cell strainer (Falcon, UK).

6) The filtrate was centrifuged at 800g for 10 minutes.

7) The mixture of culture medium and enzymes were removed with a pipette leaving the cell pellet at the bottom of the centrifuge tube.

8) Lysis of the remaining erythrocytes in the cell pellet was performed by adding 10 ml of ice cold sterile water into the centrifuge tube, after 30 seconds 15 ml of ice cold PBS was added into the mixture. The mixture was further centrifuged at 800g for 10 minutes.

9) The water and PBS mixture was removed from the centrifuge tube leaving the cell pellet behind.

10) The cell pellet was resuspended with 1ml of culture medium.

11) The concentration of the resuspended cells was measured by cell counts using the haemocytometer and trypan blue staining to determine the proportion of viable cells remaining.

12) The cells were placed into a 24 wells culture dish at a concentration of 1 x 10^5 cells per well with 2 ml of supplemented culture medium (Section 4.7).

13) The cells were cultured for 14 days at 37°C and 5% CO_2.

14) The cells were exposed to various culture conditions depending on the experiment.

The cell culture technique was previously described by Sabokbar et al (167).

4.3 Preparation of interface membrane pieces for tissue culture

1) The explantation and transport of the membrane was as described in section 4.1.

2) The interface membrane was washed thoroughly with warm (37°C) phosphate buffer saline (PBS) to remove all the blood clots. This process was repeated between 5 to 6 times depending on the amount of blood clots present.

3) The interface membrane was cut into1mm cubed pieces with sterile scalpels and scissors.

4) Multiple small pieces of interface membrane tissue were placed into a 24 well tissue culture dish for tissue culture. Each well contained 2mg of wet tissue weight.

5) The tissues were cultured in alpha MEM (Gibco BRL, Paisley, UK) culture medium for 14 days at 37°C and 5% CO_2.

4.4 Preparation of radioactive calvaria

1) Project licence [Animals (Scientific procedures) Act 1986] was obtained from the home office.

2) Ca^{45} 1mCi in the form of calcium chloride was purchased from Amersham Life Science Ltd. UK.

3) Hypodermic syringes each containing a volume of 0.05ml $Ca^{45}Cl$ (0.005mCi) was prepared from the radioactive source of 1mCi of Ca^{45} by repeated dilution with normal saline.

4) Time mated CD1 mice were used in this project.

5) The peritoneal cavity of Newborn CD1 mice were injected with 0.005mCi of Ca^{45} on day 3 and 5 with hypodermic needles.

6) After two injections the total dose per mouse was 0.01mCi.

7) The injected mice were terminated on day 6.

8) The calvaria were harvested by careful dissection. The calvaria were further halved by cutting along the median suture with a sterile scalpel and the parietal bone from each hemicalvaria was removed and used in the experiment. All the soft tissue was thoroughly removed from the parietal bone.

9) The radioactive parietal bone was kept in a freezer at –20°C.

10) Prior to use the radioactive parietal bone was subjected to 3 cycles of freeze and thaw to devitalised the cells in the parietal bone.

11) A punch biopsy knife was used to core out two 4mm bone discs from each parietal bone.

12) The pair of bone discs from the same parietal bone was used for test-control comparison in all the experiments.

13) Previous experiments have shown no difference in the radioactivity between the left and right parietal bone of the same mouse (110). In the latter part of the experiment three 3mm bone discs from the same mouse was used for three way comparisons.

4.5 Preparation of defatted devitalised bone fragments

1) Bone off cuts produced during femur and tibia preparations were taken from patients undergoing total knee replacement. All patients were consented to donate their bone for research pre operatively.

2) The bones were processed aseptically in the laboratory.

3) Using a sterile small bone nibbler the cancellous bone around the subchondral region of the tibia and femoral off cuts were removed. Most of the bone fragments obtained by this method were around 3 – 5 mm in size.

4) The bony fragments were stored in a – 80°C freezer.

5) Prior to use for experiment, the bone fragments underwent 3 cycles of freezing and thawing to devitalise the cells.

6) After the freezing / thawing cycles, the bone fragments were defatted by washing the bone with copious amount of sterile water and PBS.

4.6 Preparation of bone powder

1) Bone off cuts produced during femur and tibia preparations were taken from patients undergoing total knee replacement. All patients were consented to donate their bone for research pre operatively.

2) The bones were processed aseptically in the laboratory.

3) Using a sterile small bone nibbler the cancellous bone around the subchondral region of the tibia and femoral off cuts were removed. Most of the bone fragments obtained by this method were around 3 – 5 mm in size.

4) The bony fragments were stored in a – 80°C freezer.

5) Prior to use for experiment the bone fragments underwent 3 cycles of freezing and thawing to devitalise the cells.

6) The bone fragments were defatted by immersing the bone into 1:1 (v/v) chloroform: methanol solution for 48 hours.

7) The defatted bone fragments were washed thoroughly with sterile water and dried in sterile airing cabinet.

8) The dry defatted bone fragments were powdered using a freezer mill (Spex mill, USA) to prevent denaturization of collagen in the bone.

9) The powdered bone was sieved with a series of sieves to obtain the desirable particle size for the experiment (125 to 500 micrometer).

10) The bone powder was kept in a freezer at 80°C.

11) Prior to use the bone powder was defrosted and followed by sterilization using UV light (254nm) for 20 minutes.

4.7 Supplemented culture medium

The supplemented culture medium used in this experiments consists of Alpha MEM medium (Gibco BRL, Paisley, UK), 100 IUml of penicillin (Sigma UK), 10 mcg/ml of Streptomycin (Sigma, UK), 10 mM of 1-Glutamine (Sigma, UK), 10% fetal calf serum (FCS (Gibco BRL, Paisley, UK)), 5000ng/ml of Receptor activator N-Kappa Ligand (RANKL) (Peprotech, UK) , 1250 ng/ml of M-CSF (Peprotech, UK), 10^{-7} M of 1,25 DihydroxyVitamin D3 (Sigma, UK) and 10^{-6} M of hydrocortisone (Sigma, UK) (168).

4.8 Preparation of doxycycline culture medium

The supplemented medium was used and chemically pure doxycycline (Pfizer, UK) was added to the supplemented culture medium. The final concentration of doxycycline in the medium was 15 mcg/ml.

The mean serum concentration of doxycycline when taken at a dose of 200mg a day levels out at 1.5 mcg/ml (117). This is the recommended clinical dose for its antibiotic effect. In our study the concentration of doxycycline was set at 15 mcg/ml based on the ability of bone to concentrate doxycycline (173).

4.9 Preparation of fluvastatin culture medium

Fluvastatin powder (Norvatis, UK) was initially dissolved in 95% pure ethanol. This was followed by mixing the fluvastatin with the supplemented medium. In this experiment 20micromoles/ litre of fluvastatin were used in cell culture.

Previous study showed that fluvastatin at a concentration between 1 – 50 micromolar inhibits MMP-9 activity in human monocyte derived macrophages. In addition, fluvastatin can inhibit MMP-9 secretion in a concentration dependent manner (133). In this experiment the concentration of fluvastatin was set at 20 micromolar because this will give us the best chance to detect the inhibitory effect of fluvastatin on MMPs at the same time to minimise the possibility of cell toxicicty effect.

4.10 Supernatant sampling

1) In the preliminary and doxycycline experiments, supernatants of the culture wells were sampled using a pipette on day 3,7, 10 and 14. The same amount of medium was replenished at the same time.

2) In the fluvastatin experiment, supernatants of the culture wells were sampled using a pipette on day 7, 10 and 14. The same amount of medium was replenished at the same time.

3) Each supernatant sample was divided into 2, one half for scintillation counting and the other for NTx assay.

4.11 Bottom sampling and decalcification of radioactive calvaria with hydrochloric acid

1) After the final supernatant sampling at day 14, the radioactive calvaria was removed from the bottom of the well.

2) The calvaria was rinsed with sterile water to remove all the adherent cells.

3) 0.25 ml of 5% trypsin was added to the culture well with the remaining culture medium. This was incubated for 1 hour at 37°C.

4) After incubation the bottom of the well was scraped to release all the cells.

5) A 0.5ml sample was removed from the well for scintillation counting.

6) The washed calvaria was placed into a vial containing 3 ml of 1M of hydrochloric acid. This was left at room temperature for 3 days.

7) At day 3, a sample of 0.5ml of HCl was removed from the vial for scintillation counting to determine the remaining radioactive calcium in the disc.

4.12 Cell viability assessment using trypan blue

1) After removal of the last supernatant sample at the end of the cell culture the cells remaining at the bottom of the well were trypsinised to free the adhered cells.

2) An alliquot of Trypan blue (0.4g diluted in 100ml of normal saline) was added to the trypsinised cells.

3) A small sample of trypan blue stained cells was transferred to a clean haemocytometer with a pipette.

4) Cell counting was done using a light microscope at low power.

5) Viable and non-viable cells were counted in both halves of the chamber in the $1mm^2$ areas. This counting procedure was repeated 3 times and the average count was used for the percentage of viability count. The viable cells stayed small, round and refractile and the non-viable cells became swollen, larger and dark blue.

6) The % viability was derived from viable cell count/ total cell count x 100%. The total cell count = viable cell count + non-viable cell count.

(169)

4.13 Scintillation counting

1) A 0.5ml sample was taken from the incubation well with a pipette.

2) The sample was transferred into a scintillation vials.

3) 4.5 ml of scintillation fluid (Optiphase HiSafe 3, Fisher Scientific, UK) was added to the sample.

4) The vial was gently shaken to ensure the sample and scintillation fluid were mixed.

5) The scintillation vial was placed into the scintillation counter (Packard 1500, TRI-CARB Liquid Scintillation Analyzer, Packard, IL, USA).

6) Each vial was read three times and each reading was one minute in duration.

7) The average of the 3 counts per minute (cpm) was taken as the final reading.

8) The samples from each set of experiment were analysed on the same day to ensure that the half-life of the radioisotopes does not influence the results.

4.14 N- telopeptide assay

1) Commercial ELISA kit (Osteomark NTx serum, Ostex international, USA) was used to measure N-telopeptides of type 1 collagen (NTx).

2) A 0.5ml sample was taken from the incubation well with a pipette.

3) The samples were stored in a -40°C freezer.

4) The samples were analysed in batches. Each batch consists of 96 samples.

5) Prior to analysis the specimens and the kit component were allowed to equilibrate to room temperature (20 - 25 °C).

6) The specimens and the NTx assay were processed by following the manufacturer instructions.

7) Calibrators and controls solutions were provided by the manufacturer in the kit.

8) 100µL of test samples, calibrators and controls solutions were placed into the antigen coated 96 wells plate.

9) 100µL of working strength conjugate solution was placed into each well with a multichannel pipette.

10) A plate sealer was applied and the plate was swirled gently for 15 – 20 seconds.

11) The plate was incubated at room temperature (20 -25 °C) for 90 minutes.

12) At the end of the incubation period, the plate sealer was carefully discarded and each well was washed with working strength wash solution. The wash process was repeated 5 times. Wash volume was roughly 350µL per wash cycle.

13) Following the wash, 200µL of diluted chromogen reagent and buffered substrate were added to each well.

14) A new plate sealer was applied and the plate was incubated at room temperature (20 -25 °C) for a 30 minutes.

15) At the end of the incubation period the plate sealer was removed and 100µL of stopping reagent were added into the wells.

16) The plate was allowed to sit for a further 5 minutes at room temperature.

17) The absorbance values of the test specimen, calibrator and samples were read using a microwell plate reader within 30 minutes of adding the stopping reagent.

18) The concentration values of controls and test specimens were determined from the calibration curve and the values were expressed in nanomoles BCE/L.

BCE = Bone Collagen Equivalents.

4.15 Deoxypyrodinoline assay

1) Commercial ELISA kit (Pyrilinks, Metra Biosystems Inc, USA) was used to measure Deoxypyrodinoline crosslinks of type 1 collagen (Dpyr).

2) A 0.5ml sample was taken from the incubation well with a pipette.

3) The samples were stored in a -40°C freezer.

4) The samples were analysed in batches. Each batch consists of 96 samples.

5) Prior to analysis the specimens and the kit component were allowed to equilibrate to room temperature (20 - 25 °C).

6) The specimens and the Dpyr assay were processed by following the manufacturer instructions.

7) Calibrators and controls solutions were provided by the manufacturer in the kit.

8) 50µL of test samples, calibrators and controls solutions were diluted with 450µL of assay buffer provided by the manufacturer.

9) 50µL of the diluted test samples, calibrators and controls were added into the antigen coated 96 wells plate.

10) 100µL of working strength conjugate solution was placed into each well with a multichannel pipette.

SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN

12) At the end of the incubation period, the plate sealer was carefully discarded and each well was washed with working strength wash solution. The wash process was repeated 5 times. Wash volume was roughly 350µL per wash cycle.

13) Following the wash, 200µL of diluted chromogen reagent and buffered substrate were added to each well.

14) A new plate sealer was applied and the plate was incubated at room temperature (20 -25 °C) for a 30 minutes.

15) At the end of the incubation period the plate sealer was removed and 100µL of stopping reagent were added into the wells.

16) The plate was allowed to sit for a further 5 minutes at room temperature.

17) The absorbance values of the test specimen, calibrator and samples were read using a microwell plate reader within 30 minutes of adding the stopping reagent.

18) The concentration values of controls and test specimens were determined from the calibration curve and the values were expressed in nanomoles BCE/L.

BCE = Bone Collagen Equivalents.

4.15 Deoxypyrodinoline assay

1) Commercial ELISA kit (Pyrilinks, Metra Biosystems Inc, USA) was used to measure Deoxypyrodinoline crosslinks of type 1 collagen (Dpyr).

2) A 0.5ml sample was taken from the incubation well with a pipette.

3) The samples were stored in a -40°C freezer.

4) The samples were analysed in batches. Each batch consists of 96 samples.

5) Prior to analysis the specimens and the kit component were allowed to equilibrate to room temperature (20 - 25 °C).

6) The specimens and the Dpyr assay were processed by following the manufacturer instructions.

7) Calibrators and controls solutions were provided by the manufacturer in the kit.

8) 50µL of test samples, calibrators and controls solutions were diluted with 450µL of assay buffer provided by the manufacturer.

9) 50µL of the diluted test samples, calibrators and controls were added into the antigen coated 96 wells plate.

10) 100µL of working strength conjugate solution was placed into each well with a multichannel pipette.

11) A plate sealer was applied and the plate was swirled gently for 15 – 20 seconds.

12) The plate was incubated at 2 -5 °C for 120 minutes. The incubation was carried out in the dark as the calibrators, controls and conjugate solutions are light sensitive.

13) At the end of the incubation period, the plate sealer was carefully discarded and each well was washed with working strength wash solution. The wash process was repeated 3 times. Wash volume was roughly 250µL per wash cycle.

14) Following the wash, 150µL of working substrate solution was added to each well.

15) A new plate sealer was applied and the plate was incubated at room temperature (20 -25 °C) for a 60 minutes.

16) At the end of the incubation period the plate sealer was removed and 100µL of stopping reagent were added into the wells.

17) The plate was allowed to sit for a further 5 minutes at room temperature.

18) The absorbance values of the test specimen, calibrator and samples were read using a microwell plate reader within 15 minutes of adding the stopping reagent.

19) The concentration values of controls and test specimens were determined from the calibration curve and the values were expressed in nanomoles BCE/L.

BCE = Bone Collagen Equivalents.

CHAPTER 5

SEQUENTIAL EXPERIMENTAL DESIGN, RESULTS AND LESSONS LEARNED

This project spanned over a period of 3 years from the conception of the idea of MMP inhibitors as potential therapeutic agents for aseptic loosening to the actual testing of the agents in our in vitro model. As the project progressed, new problems were identified and valuable lessons were learned. The designs of the experiments were changed to incorporate previous lessons learned. To best understand why certain experiments were done, the experimental designs of all the experiments performed in this project are outlined sequentially in this chapter. At the end of the description of each experiment the results of the experiments are provided and this is followed by a brief discussion mainly concentrating on the lessons learned.

5.1 Experiment 1 : A simple in vitro model of aseptic loosening

Experimental design:

This experiment was performed at the start of the project. The aim of this experiment was to investigate if tissue cultured interface membrane pieces had the ability to resorb human bone fragments and at the same time to assess the usefulness of supernatant calcium, phosphate, N-telopeptide (NTx) and deoxypyrodinoline (Dpyr) as bone resorption markers.

The reasons for using pieces of interface membrane and human bone fragments in this model were because the interface membrane and bone granules were easier to prepare and the tissue was more physiological as it did not need to go through the process of cell extraction by enzymatic degradation. The bone trabeculae pattern in the bone fragment was intact and any bone resorption in this model would simulate the in vivo bone resorption more closely.

Human interface membrane and human bone were used in this experiment. The details of the patients from whom the interface membrane and bone samples were taken from are given in table 5.1. The interface membranes were obtained from patients as outlined in section 4.1. The interface membranes were processed as described in section 4.3. A 24 well tissue culture dish was used in this experiment and there were four experimental conditions. In the first well the

pieces of interface membrane were cultured with 3mg of human bone fragments. In the second well the pieces of interface membrane were cultured alone. In the third well the bone granules were cultured alone. In the fourth well the culture medium was cultured alone and this was to assess the background level of the potential markers . The culture medium used in this experiment was alpha MEM (Gibco, Paisley, UK) with 10% FCS (Gibco, Paisley, UK), 100 IU/ml of Penicillin (Sigma, UK), 10mcg of streptomycin (Sigma, UK) and 10 mM of l-glutamine. Each well contained 2ml of culture medium. This was cultured at 37°C and 5% CO_2 for 5 days. The supernatants were sampled on day 0 and 5 where 0.5ml of the medium samples were removed and the same amount were replenished on day 0 when the first samples were obtained. The level of calcium, phosphate and Dpyr were assessed. The samples from each patient provided enough cells to perform 3 sets of experiments hence a total of 9 experiments were performed (n=9). The level of calcium, phosphate and Dpyr were compared between the experimental conditions.

All data were analysed statistically using Minitab 10.5 software (Minitab Inc.). Student's t-test was used for analysis of two independent samples when data appeared to be approximately normally distributed and analysis of variance was correspondingly used for similar data from more than two independent samples.

Differences at a level of p = 0.05 were considered statistically significance.

Clinical details of patients undergoing revision THR from whom interface membrane was harvested.

Patient	Age (year)	Sex (F/M)	Diagnosis	Type of implant	Duration of implant (year)
1	76	F	OA	CoCr, PE, PMMA	11
2	69	F	OA	CoCr, PE, PMMA	8
3	72	M	OA	Ti, Ceramic	9

Table 5.1: Age and sex of patients, diagnosis, material of implants and duration of implants. CoCr = Cobalt Chrome, Ti = Titanium, PMMA = polymethylmethacrylate, PE = polyethylene, OA = osteoarthritis.

Results:

Supernatant calcium

The results are summarised in table 5.2.

Mean supernatant calcium (Ca) level in the interface membrane pieces and bone fragments culture system for day 0 and 5 in all the 4 experimental conditions

	Day 0 (min – max) mmol/l	Day 5 (min – max) mmol/l
B & Mem	1.98 (1.95 – 2.01)	1.18 (0.93 – 1.61)
Mem	2.01 (1.99 – 2.05)	1.90 (1.57 – 2.20)
B	1.99 (1.91 – 2.02)	0.73 (0.13 – 1.10)
Med	2.03 (2.02 – 2.06)	2.31 (2.13 – 2.67)

Table 5.2: The mean concentration and range of supernatant calcium in all the 4 experimental conditions on day 0 and 5. (n = 9)

B & Mem = pieces of interface membrane cultured with 3mg of human bone fragments.

Mem = pieces of interface membrane cultured alone.

B = bone fragments cultured alone.

Med = culture medium only.

The differences in calcium level on day 0 between all four cultures did not reach a statistically significance level (p = 0.35 ANOVA).

The differences in calcium level on day 5 between all four cultures were statistically significance (p = 0.0001 ANOVA).

The differences between day 5 (B & Mem) vs day 5 (Mem) were statistically significance ($p = 0.0001$ t-test).

The differences between day 5 (B & Mem) vs day 5 (B) were statistically significance ($p = 0.007$ t-test).

The differences between day 5 (B & Mem) vs day 5 (Med) were statistically significance ($p = 0.0001$ t-test).

Supernatant phosphate (PO_4)

The results are summarised in table 5.3.

Mean supernatant phosphate (PO_4) level in the interface membrane pieces and bone fragments culture system for day 0 and 5 in all the 4 experimental conditions

	Day 0 (min – max) mmol/l	Day 5 (min – max) mmol/l
B & Mem	1.15 (1.08 – 1.34)	1.17 (0.75 – 1.82)
Mem	1.17 (1.10 – 1.39)	1.53 (1.08 – 2.04)
B	1.09 (1.01 – 1.14)	0.65 (0.57 – 0.79)
Med	1.21 (1.09 – 1.69)	1.14 (1.11 – 1.17)

Table 5.3: The mean concentration and range of supernatant phosphate in all the 4 experimental conditions on day 0 and 5. (n = 9)

B & Mem = pieces of interface membrane cultured with 3mg of human bone fragments.

Mem = pieces of interface membrane cultured alone.

B = bone fragments cultured alone.

Med = culture medium only.

The differences in phosphate level on day 0 between all four cultures did not reach a statistically significance level (p = 0.28 ANOVA).

The differences in phosphate level on day 5 between all four cultures were statistically significance (p = 0.0001 ANOVA).

The differences between day 5 (B & Mem) vs day 5 (Mem) were not statistically significance (p = 0.064 t-test).

The differences between day 5 (B & Mem) vs day 5 (B) were statistically significance ($p = 0.004$ t-test).

The differences between day 5 (B & Mem) vs day 5 (Med) were not statistically significance ($p = 0.80$ t-test).

Supernatant deoxypyrodinoline (Dpyr)

The results are summarised in table 5.4.

Mean supernatant deoxypyrodinoline (Dpyr) level in the interface membrane pieces and bone fragments culture system for day 0 and 5 in all the 4 experimental conditions

	Day 0 (min – max) nmol/l	Day 5 (min – max) nmol/l
B & Mem	3.02 (1.90 – 3.80)	4.06 (3.10 – 5.30)
Mem	3.02 (2.20 – 3.60)	4.45 (3.20 – 5.90)
B	2.48 (2.00 – 3.40)	2.87 (1.80 – 3.70)
Med	2.27 (1.80 – 2.50)	2.57 (2.10 – 3.10)

Table 5.4: The mean concentration and range of supernatant phosphate in all the 4 experimental conditions on day 0 and 5. (n = 9)

B & Mem = pieces of interface membrane cultured with 3mg of human bone fragments.

Mem = pieces of interface membrane cultured alone.

B = bone fragments cultured alone.

Med = culture medium only.

The differences in Dpyr level on day 0 between all four cultures did not reach a statistically significance level (p = 0.11 ANOVA).

The differences in Dpyr level on day 5 between all four cultures were statistically significance (p = 0.004 ANOVA).

The differences between day 5 (B & Mem) vs day 5 (Mem) were not statistically significance (p = 0.67 t-test).

The differences between day 5 (B & Mem) vs day 5 (B) were not statistically significance ($p = 0.06$ t-test).

The differences between day 5 (B & Mem) vs day 5 (Med) were statistically significance ($p = 0.015$ t-test).

Discussion:

The inorganic matrix of the bone is composed of hydroxyapatite crystal. The chemical composition of hydroxyapatite is represented by the formula $Ca_{10}(PO_4)_6(OH)_2$. The hypothesis was that calcium (Ca^{2+}) and phosphate (PO_4) would be released during bone resorption. The calcium and phosphate levels in the medium would rise proportionally to the bone resorption activity. The culture well containing pieces of membrane and bone fragments was expected to show the highest level of calcium and phosphate if bone resorption was present and the calcium and phosphate levels in all the other wells were expected to remain more or less the same.

The collagen cross-linking amino acid, deoxypyrodinoline, has been used extensively in research as a bone resorption marker (144, 145). Deoxypyrodinoline is released during bone resoprtion and this was expected to rise if active bone resorption was present.

As expected for all the 3 markers (calcium, phosphate and deoxypyrodinoline), the day 0 results between groups were not significant. Bone resorption was not expected straight away at the beginning of the culture and this explained the non-significant

results. The day 0 results showed a slightly high level of background Dpyr possibly eluting from the interface membrane tissue. This may be explained by the close proximity of the interface membrane to the metabolically active bone prior to explantation from the patients.

The day 5 supernatant calcium level showed significant differences between the four experimental conditions. The culture well containing bone only (mean = 0.70 mmol/l) had the lowest calcium level followed by bone and membrane (1.08 mmol/l), membrane only (1.82 mmol/l), and medium only (2.17 mmol/l).

These results were unexpected. We did not expect the presumed dead bone to absorb calcium. The explanation for the drop in calcium could be the bone was not dead but alive and forming bone, or enzymes from dead cells were forming hydroxyapatite or the formation of calcium precipitate at the bottom of the well.

The result for day 5 phosphate level was disappointing. Comparison between groups (membrane & bone vs. membrane only, membrane & bone vs. medium only) were not statistically significant. The lowest level of phosphate was found in the bone only culture. This did reach a statistically significant level when compared with the membrane & bone culture. The phosphate level in the bone & membrane culture was more or less the same as the level found in the medium only culture. The explanation for the lowest phosphate level in the bone only culture again suggest freezed / thawed bone

may be alive, or the leaked enzymes are actively creating hydroxyapatite.

Generally Dpyr levels were greater when membrane was present both day 0 and day 5, although the difference were greater in day 5. This may represent elution from the membrane as Dpyr will be higher in all tissue of aseptic loosening or it could mean Dpyr is produced directly by the membrane. This however is contrary to the finding of Apone et al (155).

The overall results of this experiment were interesting in their own right but disappointing as none of the potential bone resorption marker tested in this experiment showed any potential for an in vitro model bone resorption marker. In fact it raises more questions than answers.

Lessons learned

The in vitro model of aseptic loosening used in this experiment was a very simple one. Since all the tested bone resorption markers were negative. This model does not explain whether the negative results was due to lack of bone resorption activity or the resorption markers simply did not work even if bone resorption was present.

The source of bone resorbing cells came from the pieces of interface membrane. The problems associated with using pieces of membranes were:

1) Difficulty in assessing whether the membrane is cellular or not. This could lead to uneven distribution of bone resorbing cells in each well.
2) Difficulty in assessing the viability of the cells / membrane.

The day 5 supernatant calcium levels in this experiment were lower in the bone only and bone & membrane culture. This was completely opposite to what was expected. Unfortunately this model does not allow us to trace the calcium to explain the reason behind this.

The next experiment was intended to answer the questions raised from this experiment. This include:

1) Can the cells from interface membrane resorb bone?
2) Can the established murine radiolabelled bone model be used to detect bone resorption?

3) As Dpyr possibly not locally produced bone marker would NTx be any better?

4) Can the drop in supernatant calcium level be explained by tracing the movement of the calcium using radioactive calcium?

5) Can N-Tx be used as an in vitro bone resorption marker as well as radioactive calcium?

5.2 Experiment 2: Can NTx be used as an in vitro debris mediated bone resorption marker?

Experimental design:

As experiment 1 had been unsuccessful in proving bone resorption occurred. The second experiment was again designed to investigate whether human interface membrane cells from aseptically loosened total hip arthroplasty had the ability to resorb bone and on this occasion investigating an alternative in vitro bone resorption marker, namely NTx, and comparing against an established bone marker, namely ^{45}Ca radiolabelled mouse (166, 110).

Four newborn CD1 mice were used in this experiment. ^{45}Ca were injected into the newborn mice and radiolabelled bone discs were obtained. The procedure is outlined in section 4.4.

Interface membranes from 4 aseptically loosened total hip replacements were obtained during revision surgery as outlined in section 4.1. Clinical details of the patients are shown in table 5.5. Cells were isolated from the interface membrane by enzyme digestion. The procedure is described in section 4.2.

There were two experimental conditions in this study. The test culture consisted of 1 x 10^5 interface membrane cells with a radiolabelled bone disc and 2ml of supplemented culture medium (see section 4.7). The control culture consisted of a radiolabelled bone disc with 2ml of supplemented culture medium only. The cell

culture was done in a 24 well cell culture dish (NUNCLON, Nalge Nunc International) and culture condition was set at 37°C with 5% CO_2. The test and control cultures were incubated for 14 days. Supernatants were sampled on day 3, 7, 10 and 14 as described in section 4.10. Each supernatant sample was divided equally for scintillation counting and NTx assay. These procedures are outlined in section 4.13 and 4.14.

On day 14 after the last supernatant sample was taken, the bone disc at the bottom of the culture well was removed and dissolved in acid to determine the total remaining ^{45}Ca in the bone disc. At the same time bottom sampling of the culture well was performed to determine the remaining ^{45}Ca at the bottom of the well. The bottom sampling and decalcification of bone disc procedures are described in section 4.11. In this experiment each patient provided enough cells to perform 2 sets of experiment hence a total of 8 sets of experiment were performed (n = 8).

All data were analysed statistically using Minitab 10.5 software (Minitab Inc.). ANOVA (General Linear Model) and paired t test were used for the analysis of data. Differences in the mean values at a level of $P < 0.05$ were considered significant. Statistical recommendations were provided by the Department of Epidemiology, University of Leicester.

Clinical details of patients undergoing revision THR from whom interface membrane was harvested.

Patient	Age (year)	Sex (f/m)	Diagnosis	Type of implant	Duration of implant (year)
1	61	f	OA	CoCr, Ceramic	9
2	84	f	OA	CoCr, PE, PMMA	13
3	54	f	OA	CoCr, PE, PMMA	8
4	61	f	OA	CoCr, PE, PMMA	7

Table 5.5: Age and sex of patients, diagnosis, material of implants and duration of implants. CoCr = Cobalt Chrome, Ti = Titanium, PMMA = polymethylmethacrylate,
PE = polyethylene, OA = osteoarthritis

Results:

For the measurement of supernatant ^{45}Ca, total ^{45}Ca remaining in the bone disc and ^{45}Ca remaining at the bottom of the well the results were expressed as scintillation count ratios of the bone disc exposed to cells (BC) over the bone disc only (B). For the serial measurements of NTx the results were expressed as NTx concentration ratios of the bone disc exposed to cells (BC) over the bone disc only (B). In this study a total of eight paired bone discs were used. The results were as follow:

Supernatant ^{45}Calcium

The mean ratio of scintillation counts (BC/B) at day 3, 7, 10 and 14 were 0.83 (range = 0.71- 0.95), 0.88 (0.70 – 1.04), 0.97 (0.77 – 1.11) and 1.08 (0.86 – 1.46) respectively. Table 5.6 shows the mean scintillation counts for the eight paired bone discs. The scintillation count ratio showed an increase of supernatant ^{45}Calcium release with time and this is shown graphically in figure 5.1 ($p = 0.0001$, ANOVA General Linear Model).

The effect of interface membrane cells on bone – ^{45}Ca released into supernatant

Paired bone discs	Supernatant scintillation count – day 3 (cpm)			Supernatant scintillation count – day 7 (cpm)			Supernatant scintillation count – day 10 (cpm)			Supernatant scintillation count – day 14 (cpm)		
	b	bc	bc/ b	b	bc	bc/ b	B	bc	bc/ b	b	bc	bc/ b
Pair 1	4161	3578	**0.86**	3794	3379	**0.89**	1931	1882	**0.97**	1496	1493	**1.00**
Pair 2	3787	3268	**0.86**	3367	2852	**0.85**	1564	1421	**0.91**	1293	1311	**1.01**
Pair 3	3184	2275	**0.71**	3001	2114	**0.70**	1792	1381	**0.77**	1060	967	**0.91**
Pair 4	2655	2016	**0.76**	1919	1995	**1.04**	1156	1245	**1.08**	644	941	**1.46**
Pair 5	1465	1375	**0.94**	1376	1343	**0.98**	810	877	**1.08**	552	643	**1.16**
Pair 6	1761	1322	**0.75**	1615	1226	**0.76**	952	816	**0.86**	646	572	**0.89**
Pair 7	1036	860	**0.83**	847	715	**0.84**	452	502	**1.11**	268	315	**1.18**
Pair 8	984	932	**0.95**	784	735	**0.94**	429	433	**1.01**	288	305	**1.06**
Mean ratio bc/b	**0.83**			**0.88**			**0.97**			**1.08**		

Table 5.6: Scintillation counts and ratio of supernatant ^{45}Ca on day 3,7, 10 and 14 from bone & cells culture and bone culture alone.

Bone only = b, bone & cells = bc and ratio of scintillation count derived from bc/b ($p = 0.0001$, ANOVA General Linear Model).

The effect of interface membrane cells on bone – supernatant ^{45}Ca (graphic summary)

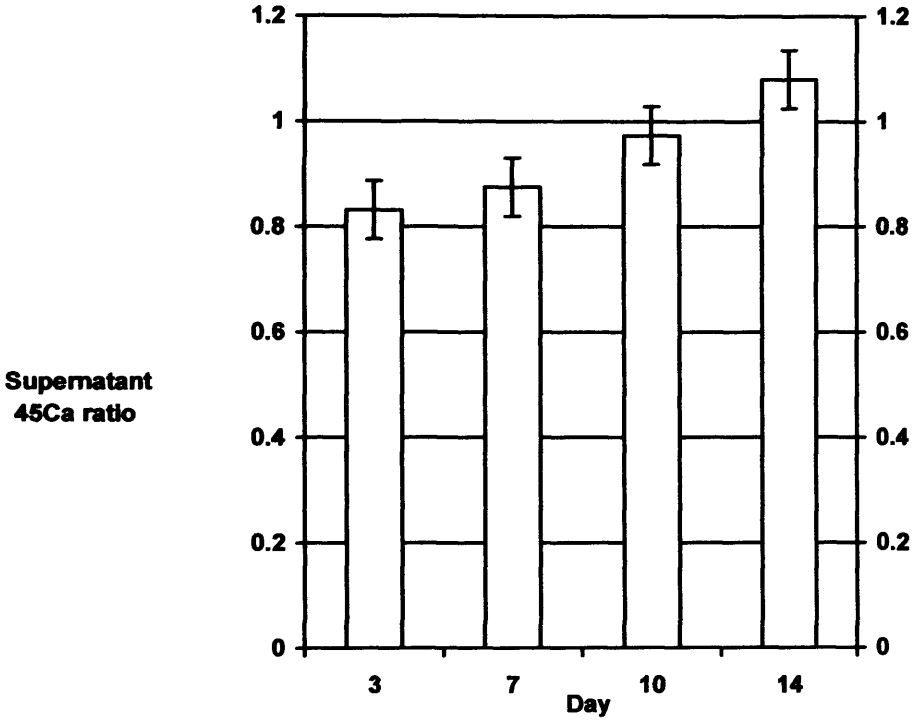


Figure 5.1: Graph showing the mean ratios of scintillation counts from supernatant of bone & cells culture and bone culture alone. Supernatant ^{45}Ca ratio derived from bone & cells / bone only. Error bar $\pm$ SEM.

Total ^{45}Calcium remaining in bone discs

The mean ratio of scintillation counts of the total ^{45}Calcium remaining in the bone discs at the completion of culture (BC/B) was 0.81 (range = 0.59 – 0.94). The mean scintillation counts are presented in table 5.7. The results show 19% more bone remaining in the absence of cells compared with presence of cells ($p = 0.02$, paired t test). This demonstrates bone resorption by cells in this model.

The effect of interface membrane cells on bone – ^{45}Ca remaining in the standardised bone samples.

Paired bone discs	^{45}Ca remaining in bone discs (cpm)		
	b	bc	bc/b
Pair 1	45942	36074	**0.79**
Pair 2	40258	27626	**0.69**
Pair 3	37214	21826	**0.59**
Pair 4	23303	18853	**0.81**
Pair 5	14992	13875	**0.93**
Pair 6	17496	13846	**0.79**
Pair 7	8036	7471	**0.93**
Pair 8	7444	7038	**0.95**
Mean ratio bc/b			**0.81**

Table 5.7: The scintillation counts and ratio of ^{45}Ca remaining in the bone discs at the conclusion of culture from culture of bones & cells vs. bone only ($p = 0.02$, paired t test).

Bone only = b, bone and cells = bc and ratio of scintillation count derived from bc/b.

^{45} Calcium at the bottom of culture wells

The mean ratio of scintillation counts of ^{45}Calcium remaining at the bottom of the well at the completion of culture (BC/B) was 1.80 (range = 1.03 – 3.85) ($p = 0.02$, paired t test). The mean scintillation counts are presented in table 5.8. This showed more ^{45}Calcium was present at the bottom of the culture wells containing interface membrane cells. The increased ^{45}Calcium at the bottom of the wells containing cells could be due to either an increased in calcium uptake by the cells or formation of an organic calcium precipitate.

The accumulation of ^{45}Ca at the bottom of culture wells with cells

Paired bone discs	^{45}Ca remaining at the bottom of culture wells (cpm)		
	b	bc	bc/b
Pair 1	842	1702	**2.02**
Pair 2	983	1560	**1.59**
Pair 3	295	1135	**3.85**
Pair 4	556	1049	**1.89**
Pair 5	335	471	**1.41**
Pair 6	416	496	**1.19**
Pair 7	173	252	**1.46**
Pair 8	170	176	**1.03**
Mean ratio bc/b			**1.80**

Table 5.8: The scintillation count and ratio of ^{45} Ca remaining at the bottom of the culture wells on day 14 from culture of bones & cells vs. bone alone ($p = 0.02$, paired t test).

Bone only = b, bone and cells = bc and ratio of scintillation count derived from bc/b.

Supernatant NTx

The mean ratio of supernatant NTx concentrations (BC/B) at day 3, 7, 10 and 14 were 1.06 (range = 0.79 – 1.33), 1.21 (0.61 – 2.09), 1.41 (1.04 – 2.18) and 1.40 (1.03 – 2.05) respectively ($p = 0.03$, ANOVA General Linear Model). This is summarised in figure 5.2. The mean concentrations of supernatant NTx are presented in table 5.9.

The effect of interface membrane cells on bone – NTx released into supernatant

Paired bone discs	Supernatant NTx – day 3 (BCE)			Supernatant NTx – day 7 (BCE)			Supernatant NTx– day 10 (BCE)			Supernatant NTx– day 14 (BCE)		
	b	bc	bc/ b	b	bc	bc/ b	b	bc	bc/ b	b	bc	bc/ b
Pair 1	175	233	**1.33**	265	184	**0.69**	82	179	**2.18**	113	232	**2.05**
Pair 2	147	116	**0.79**	103	138	**1.34**	79	161	**2.04**	178	277	**1.56**
Pair 3	421	402	**0.95**	182	381	**2.09**	220	351	**1.59**	140	239	**1.71**
Pair 4	366	372	**1.02**	306	185	**0.61**	289	346	**1.20**	190	270	**1.42**
Pair 5	272	336	**1.24**	255	336	**1.32**	294	313	**1.06**	281	289	**1.03**
Pair 6	279	298	**1.07**	222	299	**1.35**	301	314	**1.04**	208	245	**1.18**
Pair 7	319	339	**1.06**	291	354	**1.22**	270	322	**1.19**	318	336	**1.06**
Pair 8	311	311	**1.00**	236	247	**1.05**	281	298	**1.06**	266	324	**1.22**
Mean ratio bc/b	**1.06**			**1.21**			**1.41**			**1.40**		

Table 5.9: Concentrations and ratios (bc/b)of supernatant NTx on day 3,7, 10 and 14 from bone & cells culture and bone culture alone. Bone only = b, bone & cells = bc and ratio of scintillation count derived from bc/b ($p = 0.003$, ANOVA General Linear Model).
NTx concentrations are expressed as molar equivalents of bone collagen (BCE).

The effect of interface membrane cells on bone – supernatant NTx (graphic summary)

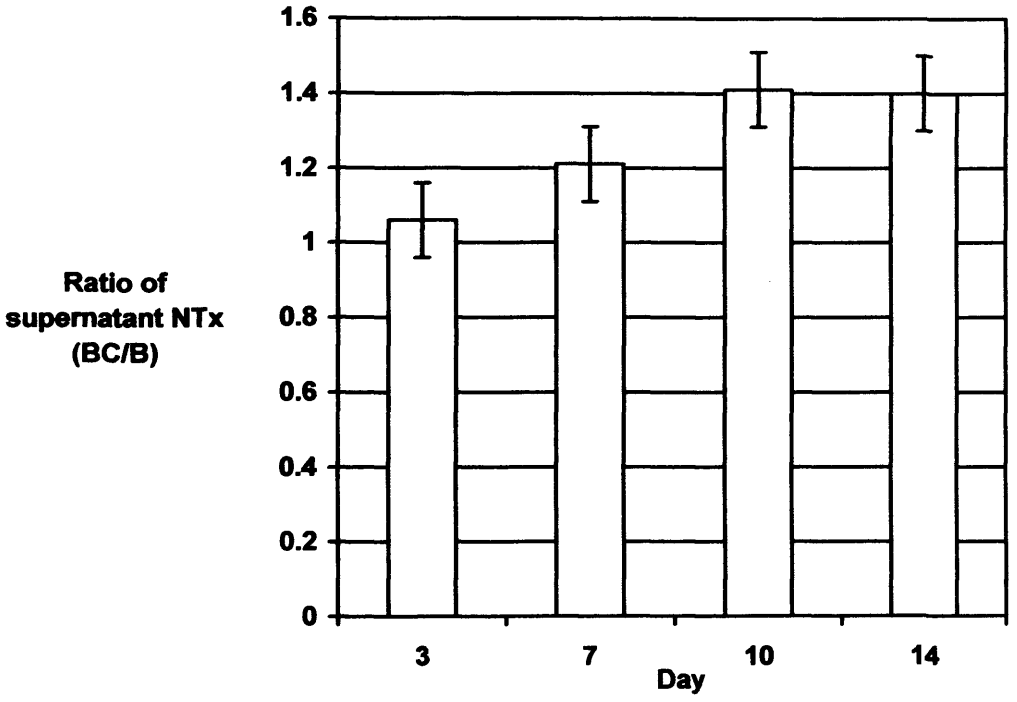


Figure 5.2: Graph showing the mean ratios of NTx from supernatant of bone & cells culture and bone culture alone. Supernatant NTx ratio derived from bone & cells / bone only. Error bar ± SEM.

Discussion:

Scintillation count ratios in the supernatant would theoretically equal 1 if there was no difference in the ^{45}Calcium release between the test and control cultures. Any rise in ratio of test over control would suggest bone resorption. Time zero assessment would theoretically produce 0/0 which would be difficult to interpret and time zero scintillation counts were not taken. The overall trend of increased supernatant ^{45}Calcium in the test cultures compared with the controls with time is shown in figure 5.1. This overall trend of increasing ratio with time is in keeping with bone resorption. The initial supernatant scintillation count ratio was less than one and initially seemed opposite to that expected. This finding was similar to the previous experiments in section 5.1. We initially thought the freeze thawed bone may not be dead and the decrease in calcium could be due to bone formation by osteoblasts in the bone. However, sampling the bottom of the wells on day 14 showed 80% higher scintillation counts in the wells with cells compared to wells with no cells. The initial drop in ratio can now be explained by either the intake of ^{45}Calcium by the cells or organic calcium precipitates. At the later part of the culture the amount of ^{45}Calcium released by bone resorption overtook the total amount of absorbed or precipitated ^{45}Calcium which resulted in a net increase of the supernatant ^{45}Calcium ratio above 1 on day 14.

In this study the paired bone discs from each test-control comparison were from the same parietal bone. The total amount of ^{45}Calcium in each bone disc should be very nearly the same at the start of the culture (110). The total ^{45}Calcium remaining in the bone discs where the discs were exposed to cells was 19% less than the controls where the bone discs were incubated alone. We believe these results confirm that bone resorption was present in our model.

Pairs 1 to 8 were ordered on numerical size and were not in time order. Any apparent decrease in scintillation counts from pair 1 to 8 is artificial.

In this study we found the supernatant NTx in the test cultures where active bone resorption took place was consistently higher than the control cultures. Hence the mean ratios of supernatant NTx concentration (BC/B) are higher than 1.0 throughout the whole culture period and it also showed an increase of NTx released with time similar to the pattern shown in the supernatant calcium 45 results. There was also a strong correlation between supernatant calcium 45 and supernatant NTx ratios ($r = 0.9$, Pearson correlation). Our results showed that supernatant NTx may be a more sensitive marker in this model as bone resorption was detected from day 3 onwards whereas bone resorption was only detected at day 14 if supernatant calcium 45 was used. Using NTx as a bone resorption marker can avoid the use of radioactive materials and it is cheaper as

the cost of radioactive waste removal can be high. Furthermore sample handling and storage are easier as NTx is a stable compound and it can be kept at a -20°C for some time prior to analysis without affecting the results (155). Measured NTx concentrations are expressed as molar equivalents of bone collagen and this allowed us to directly quantify the amount of bone resorbed, whereas the previously described bone resorption model using resorption pits as a resorption marker are only semiquantitative and does not allow serial measurement.

Unfortunately our present model yielded a high background NTx count in the control bone discs. This can be attributed to the growing bones used in this experiment where active bone remodelling was present prior to calvaria extraction and leaving a high concentration of NTx in the calvaria. In a control experiment we found the background level of NTx in the culture medium with cells from interface membrane and culture medium alone yielded no measurable NTx confirming the bone discs were the source of NTx in this experiment.

This is not the first time where NTx has been used as an in vitro bone resorption marker. Apone et al in 1997 showed NTx was generated when osteoclasts were cultured with human bone fragments and the NTx concentration correlated strongly with the resorbed area on dentin slices (165). Scheven et al in 1997 also

described a culture system where NTx was used as a bone resorption marker to monitor osteoclast activity and they found NTx concentration correlated strongly with tatrate resistant acid phosphatase (TRAP) activity which is a marker of the bone resorbing ability of cells (170).

However this is the first time NTx was shown to be a useful in vitro marker to measure bone resorption activity by cells from interface membrane of aseptically loosened THR. This is in keeping with previous clinical studies where urinary NTx levels were found to be raised in patients with aseptic loosening (163,164). These results might imply that NTx could be used to monitor the activity of aseptic loosening clinically.

This preliminary study had shown promising results and in the future we would like to develop an in vitro aseptic loosening model using mature human bone, cells from human interface membrane and NTx as the bone resorption marker. This model uses discarded human tissue, which can be easily obtained with the patients consent during joint replacement surgery and does not require the use of animal or radioactive material. Any problem from species variability would also be obviated by using an all human model.

5.3 Experiment 3: New model of aseptic loosening using interface membrane cells and powdered human bone.

Experimental design:

Previous experiments had shown that interface membrane cells had the ability to resorb bone in an established bone resorption model using radiolabelled murine bone discs. In the same experiments, NTx was shown to be a good bone resorption marker. In this experiment our aim was to establish a new in vitro model of aseptic loosening by using human interface membrane cells, powdered human bone and NTx as the resorption marker. This model will avoid the need of using live animals and radioactive materials.

In this experiment human bone powder was used instead of radiolabelled murine calvaria. Human bone was obtained from patients undergoing total knee replacement and the bone was processed into powdered bone using a freezer bone mill (Spex mill) as described in section 4.6.

Interface membranes from 4 aseptically loosened total hip replacements were obtained during revision surgery as outlined in section 4.1. Clinical details of the patients are shown in table 5.10. Cells were isolated from the interface membrane by enzyme digestion. The procedure is described in section 4.2.

The experimental conditions in this experiment were: 1) 1 x 10^5 interface membrane cells cultured with 10mg of bone powder in 2ml of supplemented culture medium (section 4.7), 2) 1 x 10^5 interface membrane cells cultured alone in 2ml of supplemented medium and 3) 10mg of bone powder cultured alone in 2ml of supplemented culture medium.

A 24 well culture dish (NUNCLON, Nalge Nunc International) was used for this experiment. Supernatant was sampled on day 3, 7, 10 and 14 as described in section 4.10. The supernatant samples were kept at 20°C and all the samples were analysed for NTx using the ELISA method as described in section 4.14.

Clinical details of patients undergoing revision THR from whom interface membrane was harvested.

Patient	Age (year)	Sex (F/M)	Diagnosis	Type of implant	Duration of implant (year)
1	56	M	OA	CoCr, PE, PMMA	7
2	82	F	OA	Cemented prosthesis	16
3	62	M	OA	CoCr, PE, PMMA	10

Table 5.10: Age and sex of patients, diagnosis, material of implants and duration of implants. CoCr = Cobalt Chrome, Ti = Titanium, PMMA = polymethylmethacrylate,
PE = polyethylene, OA = osteoarthritis

Results:

Supernatant NTx

No NTx detected in any of the samples.

Discussion:

Previous experiments had shown that NTx was produced during bone resorption in the radiolabelled murine calvaria model. Unfortunately in this experiment where human powdered bone was used instead of murine calvaria, no NTx was detected in the supernatants.

A similar model was used in the past by Apone et al, where the human bone powder was cultured with osteoclasts. In their study NTx was shown to increase with bone resorption but not deoxypyrodinoline. In their study whole human femora were used where the defatting process involved immersing the bones in a methanol/formalin mixture for 48 hours (165). In our study bone pieces measuring 2 – 4 cm were used during the defatting process. This could influence the results as the bone pieces have a larger surface area when exposed to the defatting agents. Collagen denaturalization may have occured.

Due to the spongy nature of the bone pieces, it was very difficult to get rid of all the defatting agent in the bone. It is possible that the remaining mixture of methanol/formalin was still present during the cell culture causing cell death, however the cultures were examined

by binocular microscopy and the cells were visibly motile. It is unlikely that cell death explains the negative results.

The NTx kit (Osteomark, Ostex) used in this experiment was designed for checking NTx level in human urine. The culture medium used in this experiment contained fetal calf serum and the plasma protein may have interfered with the ELISA methodology. The experiment needs to be repeated with using a plasma NTx kit.

It was a huge disappointment that this model did not work. Due to time and financial constraint it was not possible to investigate fully the reasons for the negative results. It was decided that the project should move on using the established ^{45}Ca model to test our potential therapeutic agents.

5.4 Experiment 4: Doxycycline inhibits bone resorption by interface membrane cells from aseptically loosened hip replacements.

Experimental design:

The purpose of this experiment was to investigate the ability of doxycycline to inhibit bone resorption by interface membrane cells of aseptically loosened hips using an in vitro radiolabelled mouse calvaria model.

A home office licence for animal experimentation was obtained and national guidelines for the care and use of laboratory animals were observed. Four newborn CD1 mice were used in this experiment. ^{45}Ca were injected into the newborn mice and radiolabelled bone discs were obtained (section 4.4).

Interface membranes from a further 3 aseptically loosened total hip replacements were obtained during revision surgery as outlined in section 4.1. Clinical details of these patients are shown in table 5.11. Cells were isolated from the interface membrane by enzyme digestion as described in section 4.2.

Culture dishes with 24 wells (NUNCLON, Nalge Nunc International) were used with incubation conditions set at 37°C in air with 5% of CO_2. In this experiment the test culture consisted of 1 x 10^5 interface membrane cells with bone disc and 2ml of supplemented culture medium with 15 mcg/ml of doxycycline. The preparation of

doxycycline culture medium is outlined in section 4.8. The control culture consisted of 1 x 10^5 interface membrane cells with bone disc and 2ml of supplemented culture medium but no doxycycline.

The test and control culture were incubated for 14 days. The supernatant was sampled on days 3, 7, 10 and 14. The sampling procedure is described in section 4.10. At the end of the culture period on day 14 after the final supernatant sample was taken, the bone disc was removed, washed and dissolved in acid. Scintillation counting was performed to determine the total radioactive calcium remaining in the bone disc. The decalcification process is described in section 4.11. Cell viability assessment using trypan blue staining was used to investigate possible toxicity of doxycycline on the interface membrane cells. This process is outlined in section 4.12.

All data were analysed statistically using Minitab 10.5 software (Minitab Inc.). ANOVA (General Linear Model) and paired t test were used for the analysis of data. Differences in the mean values at a level of $P < 0.05$ were considered significant. Statistical recommendations were provided by the Department of Epidemiology, University of Leicester.

Clinical details of patients undergoing revision THR from whom interface membrane was harvested (doxycycline experiment).

Patient	Age (year)	Sex (F/M)	Diagnosis	Type of implant	Duration of implant (year)
1	61	F	*OA*	CoCr, PE, PMMA	7
2	71	M	*OA*	Ti, Ceramic	13
3	84	F	*OA*	CoCr, PE, PMMA	23

Table 5.11: Age and sex of patients, diagnosis, type of implants and duration of implants. CoCr = Cobalt Chrome, Ti = Titanium, PMMA = polymethylmethacrylate,
PE = polyethylene, OA = osteoarthritis

Results:

Eight paired bone discs were used to investigate the effect of doxycycline on bone resorption. The results were as follow:

Supernatant ^{45}Calcium

The mean ratio of supernatant scintillation counts derived from bone exposed to doxycycline (Doxy) over bone disc not exposed to doxycycline (Cont) on day 3, 7, 10 and 14 were 0.94 (range = 0.81 – 1.06), 0.88 (0.76 – 1.07), 0.87 (0.65 – 1.01) and 0.81 (0.59 – 0.97) respectively ($p = 0.003$, ANOVA General Linear Model). The ratio reduced with time as shown in figure 5.3. The mean scintillation counts for the eight paired bone discs are summarised in table 5.12. There was more ^{45}Calcium released into the supernatant of the culture well with no doxycycline suggesting bone resorption was more active in the absence of doxycycline.

The effect of doxycycline on interface membrane cells ability to induce osteolysis – ^{45}Ca released into supernatant.

Paired bone discs	Supernatant scintillation count – day 3 (cpm)			Supernatant scintillation count – day 7 (cpm)			Supernatant scintillation count – day 10 (cpm)			Supernatant scintillation count – day 14 (cpm)		
	Doxy	Cont	Doxy/Cont	Doxy	Cont	Doxy/Cont	Doxy	Cont	Doxy/Cont	Doxy	Cont	Doxy/Cont
Pair 1	1260	1552	**0.81**	1019	1340	**0.76**	638	985	**0.65**	322	549	**0.59**
Pair 2	1404	1328	**1.05**	1102	1027	**1.07**	722	716	**1.00**	387	428	**0.90**
Pair 3	1040	1073	**0.97**	802	873	**0.92**	506	624	**0.81**	279	357	**0.78**
Pair 4	1193	1248	**0.96**	953	1001	**0.95**	647	695	**0.93**	320	331	**0.96**
Pair 5	795	919	**0.86**	592	666	**0.88**	422	456	**0.93**	281	297	**0.95**
Pair 6	789	927	**0.85**	590	762	**0.77**	391	477	**0.82**	238	341	**0.70**
Pair 7	1451	1434	**1.01**	920	1131	**0.81**	676	768	**0.88**	463	559	**0.83**
Pair 8	2264	2218	**1.02**	1478	1748	**0.84**	949	1049	**0.90**	574	740	**0.78**
Mean ratio doxy / cont			**0.94**			**0.88**			**0.87**			**0.81**

Table 5.12: Scintillation counts and ratio of supernatant ^{45}Ca on day 3,7, 10 and 14 from bones & cells culture with and without doxycycline.

Culture with doxycycline = doxy, culture without doxycycline (control) = cont and ratio of scintillation count derived from doxy / cont ($p = 0.003$, ANOVA General Linear Model).

The effect of doxycycline on interface membrane cells ability to induce osteolysis (graphic summary)

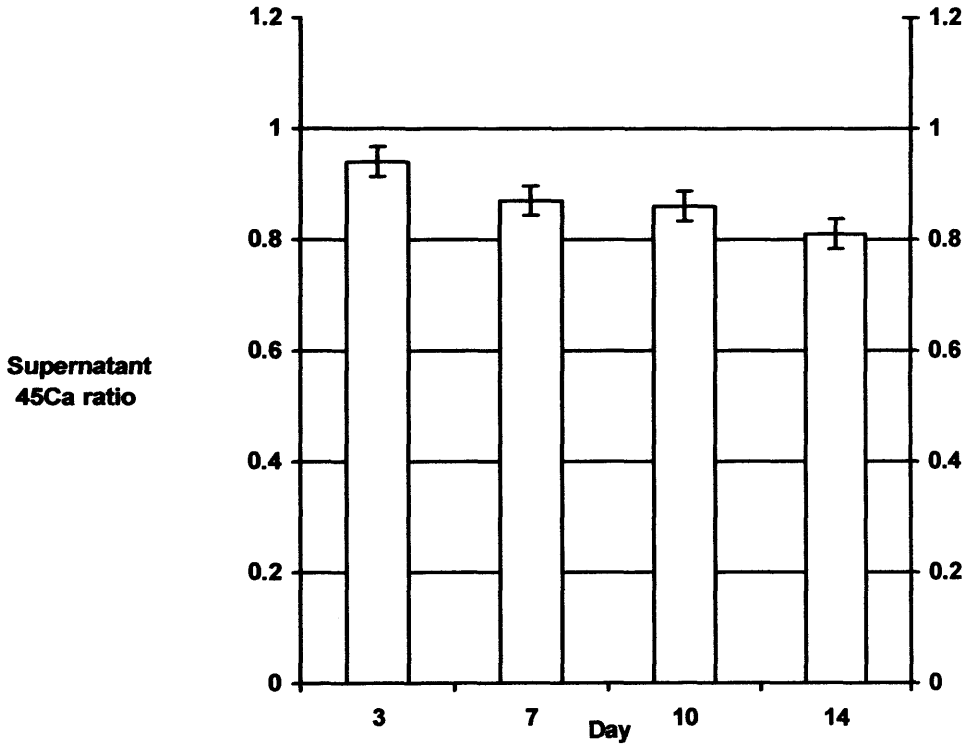


Figure 5.3: Graph showing the mean ratio of scintillation counts from supernatant of bone & cell culture with and without doxycycline. Supernatant ^{45}Ca ratio is derived from culture with doxycycline over cultures without doxycycline. Error bar $\pm$ SEM.

Total ^{45}Calcium remaining in bone disc

The mean ratio of scintillation counts of the total ^{45}Calcium remaining in the bone disc at the completion of culture (Doxy / Cont) was 1.21 (range = 1.01 – 1.49). Thus there was 21% more bone left in the doxycycline exposed bone discs compared with bone discs not exposed to doxycycline ($p = 0.02$, paired t test). This implies less bone resorption in the presence of doxycycline. The results are presented in table 5.13.

The effect of doxycycline on interface membrane cells ability to induce osteolysis - ^{45}Ca remaining in the standardised bone samples.

Paired bone discs	Scintillation counts (cpm)		
	Doxy	Cont	Doxy /Cont
Pair 1	17883	12937	**1.38**
Pair 2	12975	11416	**1.13**
Pair 3	13575	10996	**1.23**
Pair 4	17570	11762	**1.49**
Pair 5	9307	7350	**1.27**
Pair 6	8486	7578	**1.12**
Pair 7	14485	14031	**1.03**
Pair 8	16972	16763	**1.01**
Mean ratio doxy / cont			**1.21**

Table 5.13: Scintillation counts and ratios of total ^{45}Ca remaining in eight pairs of bone discs at the conclusion of 14 days culture with interface membrane cells in the presence and absence of doxycycline. Culture with doxycycline = doxy, culture without doxycycline (control) = cont and ratio of scintillation count derived from doxy / cont ($p = 0.02$, paired t test).

Cell viability

Cell viability in both the doxycycline and non doxycycline cultures were almost the same; 86% (range = 82% - 90%) in the doxycycline culture and 85% (78% - 89%) in the non doxycycline culture ($p = 0.63$, ANOVA). Hence doxycycline was not cytotoxic.

Discussion:

In this study, the scintillation counts for supernatant ^{45}Calcium in the test well (doxycycline, cells and bone disc) were consistently higher compared with the controls (cells and bone disc) throughout the whole experiment. This was reflected in the ratio of supernatant ^{45}Calcium remaining below 1 as shown in figure 5.3. Any calcium uptake or organic precipitate should be the same in test and control cultures and the confusing results seen on the bone resorption part of the study would not be expected in this latter part of the study looking at the effect of doxycycline. The total ^{45}Calcium remaining in the bone discs on day 14 was higher in the doxycycline containing culture wells compared with the wells without doxycycline (table 5.13). Thus the presence of doxycycline inhibited bone resorption by interface membrane cells. The inhibitory effect of doxycycline in this model was not due to cell toxicity as cell viability tests were similar in both cultures. It has been shown in previous studies that doxycycline can inhibit osteoclasts both in vitro (171) and in vivo (172). This is the first time where doxycycline has been shown to inhibit bone resorption by interface membrane cells. We believe MMP inhibition by doxycycline offers a possible explanation.

Orally administered tetracycline is taken up and incorporated into hydroxyapatite crystals together with calcium in the inorganic bone matrix. Upon degradation of the bone, tetracycline is released,

yielding high concentrations of localized tetracycline (173). The concentration of doxycycline in periodontal tissue was about ten times greater when compared with serum level (174, 175). The average serum concentration of doxycycline when taken at a dose of 200 mg a day level out at 1.5 mcg/ml. This is the recommended clinical dose for its antibiotic effect (117). In our study the concentration of doxycycline was set at 15 mcg/ml based on the ability of bone to concentrate doxycycline. Furthermore a concentration of as low as 5 mcg/ml has been shown to inhibit bone resorption by chick osteoclasts (176).

Previous studies have shown that pharmacological inhibition of aseptic loosening may be a realistic prospect. Doxycycline is another possible agent that could influence aseptic loosening either alone or in combination with other agents. It is relatively inexpensive with extensive clinical use as an antibiotic with minimal reported adverse reactions.

In this experiment we were unable to ascertain the magnitude of inhibition due to the design of the study. In the next experiment the experimental design will be change slightly to enable the determination of magnitude of inhibition.

5.5 Experiment 5 : Fluvastatin inhibits bone resorption by human interface membrane cells from aseptically loosened hip replacements.

The purpose of this experiment was to investigate the ability of fluvastatin to inhibit bone resorption by interface membrane cells of aseptically loosened hips using an in vitro radiolabelled mouse calvaria model.

A home office licence for animal experimentation was obtained and national guidelines for the care and use of laboratory animals were observed. Eight newborn CD1 mice were used in this experiment. ^{45}Ca was injected into the newborn mice and radiolabelled bone discs were obtained as outlined in section 4.4. In this experiment 3mm diameter bone discs were used rather than 4mm to allow 3 discs from each calvaria.

Interface membranes from 4 aseptically loosened total hip replacements were obtained during revision surgery as outlined in section 4.1. Clinical details of the patients are shown in table 5.14. Cells were isolated from the interface membrane by enzyme digestion as described in section 4.2.

Culture dishes with 24 wells (NUNCLON, Nalge Nunc International) were used with incubation conditions set at 37°C in air with 5% of CO_2 for 14 days. In this study, the experimental conditions were: 1) 1×10^5 interface membrane cells cultured with 3mm radiolabelled

bone disc and 2ml of supplemented culture medium with 20 microM of fluvastatin (Norvatis, UK). The preparation of fluvastatin culture medium is outlined in section 4.9. 2) 1 x 10^5 interface membrane cells cultured with 3mm radiolabelled bone disc and 2ml of supplemented culture medium but no fluvastatin and 3) 3mm radiolabelled bone disc cultured in supplemented culture medium. The 3 bone discs used for each set of experiments were from the same mouse to ensure similar level of ^{45}Ca at the start of the experiment.

The supernatant was sampled on days 7, 10 and 14. The sampling procedure is described in section 4.10. At the end of culture period on day 14 after the final supernatant sample was taken, the bone disc was removed, washed and dissolved in acid. Scintillation counting was performed to determine the total radioactive calcium remaining in the bone disc. The decalcification process is described in section 4.11. Cell viability assessment using trypan blue staining was used to investigate any toxicity effect of fluvastatin on the interface membrane cells. This process is outlined in section 4.12. The experiments were repeated 8 times. Patient 1 and 2 provided enough cells for the first 6 sets of experiment and patient 3 provided the cells for the remaining experiments (n = 8).

In this experiment there were 3 experimental conditions compared to only 2 experimental conditions in the doxycycline experiment. This

was to allow the determination of baseline resorption and calculation of the degree of inhibition. The results are expressed as a ratio of bone disc exposed to cells and fluvastatin over unexposed bone disc (bone disc culture only). In the control the results are expressed as a ratio of bone disc exposed to cells over unexposed bone disc (bone culture only).

Clinical details of patients undergoing revision THR from whom interface membrane was harvested for the fluvastatin experiment.

Patient	**Age (year)**	**Sex (F/M)**	**Diagnosis**	**Type of implant**	**Duration of implant (year)**
1	55	F	*OA*	CoCr, PE, PMMA	7
2	78	M	*OA*	CoCr, PE, PMMA	8
3	86	F	*OA*	CoCr, PE, PMMA	12

Table 5.14: Age and sex of patients, diagnosis, material of implants and duration of implants. CoCr = Cobalt Chrome, PMMA = polymethylmethacrylate, PE = polyethylene, OA = osteoarthritis

Results:

A total of eight experiments were performed to investigate the effect of fluvastatin on bone resorption. The results were as follow:

Supernatant ^{45}Calcium

The mean scintillation counts for each of the experimental conditions in all the eight experiments are summarised in table 5.10. The ratios of mean ^{45}Ca supernatant scintillation counts on day 7, 10 and 14 are summarised in table 5.15. Both the fluvastatin and control ratios increased with time but the culture containing fluvastatin consistently showed a lower ratio compared with control throughout the experiment. The mean ratios in the fluvastatin cultures on day 7, 10 and 14 were 0.79, 1.53 and 2.55. The mean ratios in the control cultures on day 7, 10 and 14 were 1.03, 1.81 and 3.20. Summary of results is shown in table 5.16 and fig. 5.4 ($p = 0.0001$ ANOVA, General Linear Model).

The effect of fluvastatin on interface membrane cells ability to induce osteolysis – ^{45}Ca released into supernatant.

Paired bone discs	Supernatant scintillation count – day 7 (cpm)			Supernatant scintillation count – day 10 (cpm)			Supernatant scintillation count – day 14 (cpm)		
	Flu	BC	B	Flu	BC	B	Flu	BC	B
Experiment 1	3797	4766	4738	937	1206	457	535	680	253
Experiment 2	3584	5985	5558	798	1119	816	531	664	448
Experiment 3	2432	4748	3642	1571	1788	1117	716	783	252
Experiment 4	3569	3889	3091	2204	2474	1170	958	1025	239
Experiment 5	2620	3158	4222	1818	2153	911	861	910	214
Experiment 6	2684	3018	3154	1426	1433	1260	414	567	173
Experiment 7	2196	2731	2561	1145	1466	926	410	606	141
Experiment 8	3091	3455	4171	1583	1682	985	264	550	252

Table 5.15: Scintillation counts of supernatant ^{45}Ca on day 7, 10 and 14 from bones & cells culture with fluvastatin (Flu), bone & cells culture without fluvastatin (BC) and bone culture only (B).

The effect of fluvastatin on interface membrane cells ability to induce osteolysis – ratio of ^{45}Ca released into supernatant.

	Ratio of supernatant scintillation count – day 7 (cpm)		Ratio of supernatant scintillation count – day 10 (cpm)		Ratio of supernatant scintillation count – day 14 (cpm)	
	Flu	Control	Flu	Control	Flu	Control
Experiment 1	0.80	1.01	2.00	2.60	2.1	2.7
Experiment 2	0.65	1.08	0.98	1.37	1.19	1.48
Experiment 3	0.67	1.30	1.41	1.60	2.84	3.11
Experiment 4	1.16	1.26	1.88	2.12	4.00	4.29
Experiment 5	0.62	0.75	2.00	2.36	4.02	4.25
Experiment 6	0.85	0.96	1.13	1.14	2.39	3.28
Experiment 7	0.86	1.07	1.24	1.58	2.91	4.30
Experiment 8	0.74	0.83	1.61	1.71	1.05	2.18
Mean ratios	**0.79**	**1.03**	**1.53**	**1.81**	**2.55**	**3.20**

Table 5.16: Ratio of mean ^{45}Ca supernatant scintillation counts on day 7, 10 and 14. The ratios are derived from: Flu = mean ^{45}Ca supernatant scintillation counts of bones & cells culture with fluvastatin (Flu) / bone culture only (B) and control = mean ^{45}Ca supernatant scintillation counts of bone & cells culture without fluvastatin (BC) and bone culture only (B). P = 0.0001 (ANOVA, General Linear Model).

The effect of fluvastatin on interface membrane cells ability to induce osteolysis – ^{45}Ca released into supernatant (graphic summary)

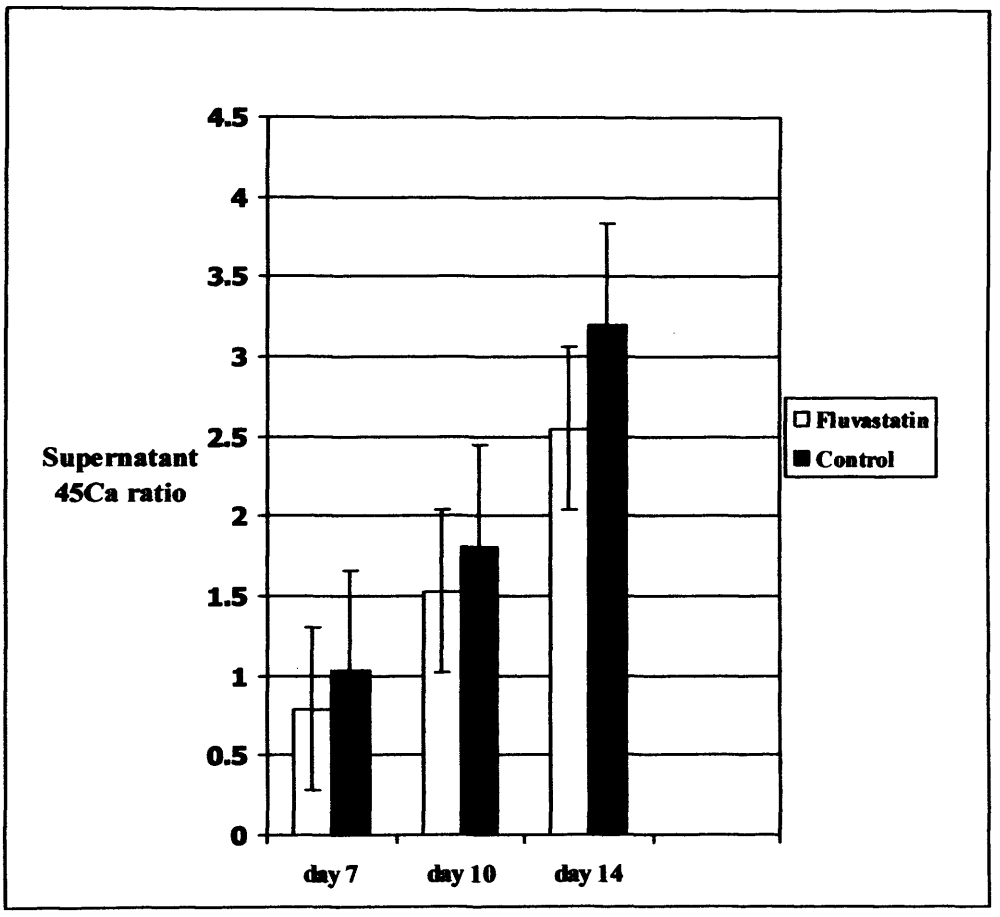


Figure 5.4: Graph showing the mean ratio of ^{45}Ca supernatant scintillation counts on day 7, 10 and 14. The ratios are derived from: Flu = mean ^{45}Ca supernatant scintillation counts of bones & cells culture with fluvastatin (Flu) / bone culture only (B) and control = mean ^{45}Ca supernatant scintillation counts of bone & cells culture without fluvastatin (BC) and bone culture only (B). P = 0.0001 (ANOVA, General Linear Model).

Total ^{45}Calcium remaining in bone disc

The mean ratio of scintillation count of the total ^{45}Calcium remaining in the bone disc at the completion of culture in the fluvastatin group was 0.87 and the control was 0.70 (p=0.01, t test). This implies 55% less bone resorption in the presence of fluvastatin. The results are presented in table 5.17.

The effect of fluvastatin on interface membrane cells ability to induce osteolysis - ^{45}Ca remaining in the standardised bone samples.

	Scintillation counts (cpm)			Ratios	
	Fluvastatin	Bone & cells	Bone only	Fluvastatin	Control
Experiment 1	23456	20970	25055	0.94	0.84
Experiment 2	20223	17459	29989	0.67	0.58
Experiment 3	16074	9901	18523	0.87	0.53
Experiment 4	15951	13594	19792	0.81	0.69
Experiment 5	15177	15300	20251	0.75	0.76
Experiment 6	16339	12464	17330	0.94	0.72
Experiment 7	11419	9754	11628	0.98	0.84
Experiment 8	17548	11393	17809	0.99	0.64
Mean ratio				**0.87**	**0.70**

Table 5.17: Scintillation counts and ratios of total ^{45}Ca remaining in bone discs at the conclusion of 14 days culture. Culture containing fluvastatin, cells and bone discs = Fluvastatin, culture containing cells and bone disc only = Bone & cells and culture containing bone disc only = Bone only. Ratio of scintillation count calculated using the following formula: Fluvastatin = fluvastatin scintillation count / Bone only scintillation count and Control = Bone & cells scintillation count / Bone only scintillation count.

Cell viability

Cell viability in both the fluvastatin and non fluvastatin cultures were almost the same; 81% (range = 69% - 90%) in the fluvastatin culture and 80% (74% - 92%) in the non fluvastatin culture ($p = 0.88$, ANOVA). Hence fluvastatin was not cytotoxic.

Discussion:

In this experiments 3 bone discs were used instead of two as previously described in the doxycycline experiments. Using the 3 discs model allows us to compare the effect of fluvastatin against baseline bone resorption by interface membrane cells and allowed the quantification of the degree of inhibition.

The results from previous experiments showed that changes in the supernatant ^{45}Ca started to rise on from day 7 onward reflecting bone resorption activity. Hence the supernatant sampling in this experiment started on day 7 instead of the previous day 3 sampling. As this experiment uses a primary cell culture system by starting the sampling on day 7 allowed the cells to settle down and established themselves in the culture system and at the same time reduced the chances of culture contamination from repeated sampling.

In this the study, the mean ratio of ^{45}Ca supernatant scintillation count in the fluvastatin culture were found to be significantly lower compared to the control ratio where no fluvastatin was present throughout the whole culture period. This means less ^{45}Ca was

released into the culture medium in the presence of fluvastatin suggesting less bone resorption activity. The inhibition of bone resorption activity by fluvastatin was further confirmed by the results of the final total ^{45}Ca remaining in the bone discs. This study showed that bone resorption was still present in the fluvastatin culture where 13% of the bone was resorbed but in the control culture 30% of the bone was resorbed. This results implied that in the presence of fluvastatin bone resorption was reduced by 55%.

In the cell viability study using trypan blue method, there were no significant difference between fluvastatin and control. This means that the inhibition of bone resorption was not due to cell toxicity.

This is the first time fluvastatin has been shown to reduced debris induced osteolysis. Unfortunately this study was not designed to determine the mechanism of action of fluvastatin. Previous studies have shown that fluvastatin can inhibit MMPs in macrophages via the mavulonic acid pathway (133). This is the most likely explanation for the anti-osteolytic property of fluvastatin observed in this study. Statins have also been shown to increase murine calvarial bone density by increasing BMP in bone (134). Though this pathway is unlikely to explain the anti-osteolytic property of fluvastatin in this study because the bone discs were devitalised in this experiment. In clinical practice this pathway could be an added bonus as

theoretically fluvastatin is not only able to inhibit bone resorption in aseptic loosening but also increase bone formation.

Fluvastatin is commonly used to reduce cholesterol and has a known safety profile (129). Based on the results from this study fluvastatin may be used in the treatment of aseptic loosening. More research is needed in the future to investigate its mechanisms of action, the optimal dose and also the timing of administration.

CHAPTER 6

THE FINAL CHAPTER

(DISCUSSION & THE FUTURE)

This project illustrated very clearly the ups and downs of scientific experiments. The first aim of the project was to develop a simple in vitro model of aseptic loosening that will allowed the testing of potential pharmacological agents against aseptic loosening. In 1969, Reynolds and Dingle, described an in vitro method to investigate bone resorption using ^{45}Ca labelled murine calvaria. They found this method to be extremely sensitive in the study of hormone induced bone resorption (166). The radiolabelled murine calvaria model had been adopted and modified by various investigators of aseptic loosening over the years. Horrowitz et al used live radiolabelled calvaria to investigate the effect of wear particle activated macrophages on bone resorption (78) and later he used a similar model to investigate the pharmacological effect of bisphosphonate on aseptic loosening (110). Yokohama et al uses dead radiolabelled calvaria to investigate the direct bone resorption activity of wear particle activated macrophages (104). This method was widely used for the investigation of bone resorption because it is relatively easy to prepare, as all the materials needed for the model are widely available. The downsides of this model are the investigator is exposed to radioactive material and it is expensive to dispose of the radioactive waste at the end of the experiment.

Quantifying bone resoprtion by counting bone resoprtion pit formation in cortical bone slices is another widely used method in

the investigation of bone resoprtion in aseptic loosening (109, 168). Unfortunately this method is only semiquantitative and it is technically difficult, and expensive to produce thin cortical bone slices with smooth surfaces.

Therefore we set out to develop a simple in vitro model to investigate bone resorption induced by interface membrane from aseptically loosened hips which addressed all the negative aspects of previous models. The first experiment involved culturing interface membrane tissue together with human bone granules. The bone resoprtion markers used in this experiment were supernatant calcium, phosphate and Dpyr. Unfortunately our simple model failed to work and this was discussed in chapter 5.

In the second experiment, cells from the interface membrane were extracted and cultured with dead ^{45}Ca radiolabelled murine bone disc. Cytokines were added to the culture medium to encourage bone resorption. ^{45}Ca was measured indirectly using scintillation counts and NTx was measured using an ELISA method. These were used as indicators of bone resorption. In this experiment, we showed bone resorption was present and NTx may be used as an alternative in vitro bone resorption marker instead of ^{45}Ca.

Using NTx as an in vitro resorption marker is not a new idea. Apone et al (165) and Scheven et al (170) had previously showed that NTx was liberated during bone resorption by osteoclasts and this was in

direct proportion to resorption activity. In this experiment, for the first time we have shown that NTx was produced during bone resorption by interface membrane cells. There was a good correlation between ^{45}Ca and NTx. The findings in this experiment are consistent with previous clinical studies which showed urinary NTx was raised in patients with aseptic loosening (163,164). Assuming NTx is produced at the bone prosthesis interface during aseptic loosening then by measuring NTx levels in patients serum or urine, it may be possible to monitor resorption activity and thus aseptic loosening.

Following the successful second experiment, the experimental model was further modified. In this experiment, the ^{45}Ca radiolabelled murine bone discs were replaced with defatted powdered human bone. NTx alone was used as a resorption marker. Unfortunately no NTx was detected in any of the samples. We were unable to explain the negative results. Unfortunately due to time and financial constraints we were unable to continue with the development of NTx in this model. Hence the established ^{45}Ca radiolabelled murine bone discs were used in subsequent experiments.

The purpose of the present study was to determine whether doxycycline and fluvastatin are capable of inhibiting bone resorption in aseptic loosening. Both agents were tested in our in vitro model of aseptic loosening.

We found the presence of doxycycline inhibited bone resorption by interface membrane cells. Unfortunately we were unable to determine the degree of inhibition as comparisons were of ratios and not absolute values. In the subsequent experiments, a three disc culture system was used instead of two. This model allowed us to determine the degree of inhibition. Fluvastatin was tested using this model. We found fluvastatin inhibited bone resorption by 55%.

We can only speculate that MMPs inhibition lead to the reduction of bone resorption as we did not measure MMPs levels. Currently, there are no approved drug therapies to treat aseptic loosening of total joint replacements. In one small clinical study of bisphosphonates (alendronate) showed a reduction in periprosthetic bone loss that normally develops early after total hip replacement (177). However, the authors pointed out that this early bone loss was probably secondary to stress-shielding rather than to wear debris induced osteolysis, since patients who had been followed for more than five years after total hip replacement and those who were waiting revision surgery because of aseptic loosening did not have a similar increase in periprosthetic bone density. It remains to be seen if bisphosphonates will be effective for the treatment of aseptic loosening.

Pentoxyfyline has been shown in a previous study to decrease the release of TNF - α from human peripheral blood monocytes exposed

ex vivo to particle debris (116). As TNF - α is involved in the pathogenesis of osteolysis and subsequent loosening of total joint arthroplasty components therefore it was indirectly concluded that pentoxyfyline may be useful in the treatment of aseptic loosening. There is no study yet to determine the effectiveness of this drug in true aseptic loosening.

As a result of the revolution in molecular biology and genetics over the last twenty years, biological therapies for osteolysis have become feasible. Elucidation of the roles of RANK and RANKL in the regulation of osteoclastogenesis has provided another therapeutic target to prevent osteolysis (67). A recent study by Ulrich-Vinther et al showed that the gene expressing the RANK protein can be transmitted to mice using adeno associated virus to act as a vector. The mice infected with the virus carrying the RANK expressing gene had the ability to inhibits debris induced osteolysis (178). The prospect of gene therapy in the fight against aseptic loosening is an interesting one, but the safety and efficacy of this technique especially in human use is still uncertain.

Our current findings of doxycycline and fluvastatin as potential therapeutic agents for the treatment of aseptic loosening merit further investigations because both drugs are widely used in clinical practice with a proven safety record for other unrelated conditions. These drugs can provide a short-term solution to the treatment of

aseptic loosening until better alternatives are found. Future studies designed include investigating the efficacy of these drugs in vivo, the mechanisms of action, optimum dosage and the effect of combining drugs and eventually testing in human aseptic loosening.

REFERENCES

1. Charnley J. Anchorage of the femoral head prosthesis to the shaft of the femur. J Bone Joint Surg [Br] 1960; 42-B:28-30.

2. Malchau H, Herberts P, Sőderman P, Oděn A. Prognosis of total hip replacement: update and validation of results from the Swedish National Hip Arthroplasty Register 1979 – 1998. 67th Annual Meeting American Academy of Orthopaedics Surgeons, 2000.

3. Gelb H, Schumacher HR, Cuckler J, Baker DG. In vivo inflammatory responses to polymethylmethacrylate particulate debris: effect of size, morphology and surface area. J Orthop Res. 1994; 12:83-92.

4. Schmalzried TP, Jasty M, Harris WH. Periprosthetic bone loss in total hip arthroplasty: polyethylene wear debris and the concept of effected joint space. J Bone Joint Surg [Am] 1992; 74-A:849-863.

5. Santavirta S, Konttinen YT, Bergroth V, Eskola A, Tallroth K, Lindholm TS. Aggressive granulomatous lesions associated with hip arthroplasty. Immunopathological studies. J Bone Joint Surg [Am] 1990; 72-A:252-258.

6. Lucht U. The Danish Hip Arthroplasty Register. Acta Orthop Scand 2000; 71(5):433-439.

7. Fender D, Harper WM, Gregg PJ. Outcome of Charnley total hip replacement across a single health region in England. The results at five years from regional arthroplasty register. J Bone Joint Surg [Br] 1999; 81-B:577-581.

8. Garelick G, Herberts P, Strőmberg C, Malchau H. Long term results of Charnley arthroplasty: a 12 – 16 year follow up study. J Arthroplasty 1994; 9:330-340.

9. Salvati EA, Wilson PD Jr, Jolley MN et al. A ten year follow up of our first one hundred consecutive Charnley total hip replacements. J Bone Joint Surg [Am] 1981; 63-A:753-767.

10. Schulte KR, Callaghan JJ, Kelley SS, Johnston RC. The outcome of Charnley total hip arthroplasty with cement after a minimum twenty-year follow up: the results of one surgeon. J Bone Joint Surg [Am] 1993; 75-A:961-975.

11. Wroblewski BM, Siney PD. Charnley low friction arthroplasty of the hip: long term results. Clin Orthop 1993; 292:191-201.

12. Tay BH, Masbah O, Razak M, Ruslan GNS. Total hip arthroplasty in Malaysia – The Universiti Kebangsaan Malaysia and Hospital Kuala Lumpur Experience. Med J Malaysia 2000; Vol 55 Suppl C:74-85.

13. Kavanagh BF, Wallrichs S, Dewitz M, Berry D, Currier B, Ilstrup D, Coventry MB. Charnley low friction arthroplasty of the hip. Twenty-year results with cement. J Arthroplasty 1994; 9(3):229-234.

14. Harris WH, Schiller AL, Scholler JM, Freiberg RA, Scott R. Extensive localized bone resorption in the femur following total joint replacement. J Bone Joint Surg [Am] 1976; 58-A:612-618.

15. Haynes DR, Rogers SD, Hay S, Pearch MJ, Howie DW. The differences in toxicity and release of bone resorbing mediators induced by titanium and cobalt-chrome alloy particles. J Bone Joint Surg [Am] 1993; 75-A:799-801.

16. Pazzaglia UE, Cecilliani L, Wilkinson MJ, Dell'Orbo C. Involvement of metal particles in loosening of metal-plastic total hip prosthesis. Arch Orthop Trauma Surg 1985; 104:164-174.

17. Boynton E, Waddel JP, Morton J, Gardiner GW. Aseptic loosening in total hip implants: the role of polyethylene wear debris. Can J Surg 1991; 34:599-605.

18. Willert HG, Bertram G, Buchhorn GH. Osteolysis in alloarthroplasty of the hip: the role of ultra high molecular weight polyethylene wear particles. Clin Orthop 1990; 258:95-107.

19. Horrowitz SM, Doty SB, Lane JM, Burstein AH. Studies of the mechanism by which the mechanical failure of polymethylmethacrylate leads to bone resorption. J Bone Joint Surg [Am] 1993; 75-A:802-813.

20. Yoon TR, Rowe SM, Jung ST, Seon KJ, Maloney WJ. Osteolysis in association with total hip arthroplasty with ceramic bearing surfaces. J Bone Joint Surg [Am] 1998; 80-A:1459-1468.

21. Kobayashi A, Freeman MAR, Bonfield W, Kadoya Y, Yamac T, Al-Saffar N, Scott G, Revell PA. Number of polyethylene particles and osteolysis in total joint replacements – a quantitative study with tissue digestion method. J Bone Joint Surg [Br] 1997; 79-B:844-848.

22. Kadoya Y, Kobayashi A, Ohashi H. Wear and osteolysis in joint replacements. Acta Orthop Scand 1998; 69 Suppl 278:1-16.

23. Gruen TA, McNeice GM, Amstuz HC. "Modes of failure" of cemented stem-type femoral components: a radiographic analysis of loosening. Clin Orthop 1979; 141:17-27.

24. Carlsson AS, Gentz C-F, Linder L. Localized bone resorption in mechanical failure of cemented total hip arthroplasties. Acta Orthop Scand 1983; 54:396-402.

25. Ferrier GM, McEvoy A, Evans CE, Andrew JG. The effect of cyclic pressure on human monocyte-derived macrophages in vitro. J Bone Joint Surg [Br] 2000; 82-B:755-759.

26. Jones LC, Frondoza C, Hungerford DS. Effect of PMMA particles and movement on an implant interface in a canine model. J Bone Joint Surg [Br] 2001; 83-B:448-458.

27. Evans EM, Freeman MAR, Miller AJ, Vernon-Roberts B. Metal sensitivity as a cause of bone necrosis and loosening of the prosthesis in total joint replacement. J Bone Joint Surg [Br] 1974; 56-B:626-629.

28. Goldring SR, Schiller AL, Roelke M, Rourke CM, O'Neill DA, Harris WH. The synovial-like membrane of the bone-cement interface in loose total hip replacement and its proposed role in bone lysis. J Bone Joint Surg [Am] 1983; 65-A:575-584.

29. Linder L, Lindberg L, Carlsson Å. Aseptic loosening of hip prostheses. A histologic and enzyme histochemical study. Clin Orthop 1983; 175:93-104.

30. Al Saffar N, Revell PA. Interleukin-1 production by activated macrophages surrounding loosened orthopaedic implants: a potential role in osteolysis. Br J of Rheumatology 1994; 33:309-316.

31. Maloney WJ, Smith RL. Periprosthetic osteolysis in total hip arthroplasty: the role of particulate wear debris. J Bone Joint Surg [Am] 1995; 77-A:1448-1461.

32. Jones LC, Frondoza C, Hungerford DS. Immunohistochemical evaluation of the interface membranes from failed cemented and uncemented acetabular components. J Bio Mat Res 1999; 48(6):889-898.

33. Goodman SB, Huie P, Song Y, Schurman D, Maloney W, Woolson S, Sibley R. Cellular profile and cytokine production at prosthetic interfaces. J Bone Joint Surg [Br] 1998; 80-B:531-539.

34. Gil-Albarova J, Lacleriga A, Barrios C, Canadell J. Lymphocyte response to PMMA in loose total hip prostheses. J Bone Joint Surg [Br] 1992; 74-B:825-830.

35. Haddad FS, Cobb AG, Bentley G, Levell NJ, Dowd PM. Hypersensitivity in aseptic loosening of total hip replacements. J Bone Joint Surg [Br] 1996; 78-B:546-549.

36. Jiranek W, Jasty M, Wang JT, Bragdon C, Wolfe F, Goldberg M, Harris WH. Tissue response to particulate polymethylmethacrylate in mice with various immune deficiencies. J Bone Joint Surg [Am] 1995; 77-A:1650-1661.

37. Sandhu J, Waddel JE, Henry M, Boynton EL. The role of T-cells in polyethylene particulate induced inflammation. J Rheumatol 1998; 25(9):1794-1799.

38. Li TF, Santavirta S, Waris V, Lassus J, Lindroos L, Xu JW, Virtannen I, Konttinen YT. No lymphokines in T cells around loosened hip prostheses. Acta Orthop Scand 2001; 72(3):241-247.

39. Athanasou NA, Quinn J, Bulstrode CJK. Resorption of bone by inflammatory cells derived from the joint capsule of hip arthroplasties. J Bone Joint Surg [Br] 1992; 74-B:57-62.

40. Kadoya Y, Al-Saffar N, Revell PA, Kobayashi A, Scott G, Freeman MAR. Bone loss in aseptic loosening – bone resorption by osteoclasts or macrophages? Trans ORS 1995 41st annual meeting, Orlando, Florida; 20:215.

41. Kadoya Y, Al-Saffar N, Revell PA, Kobayashi A. The expression of osteoclast markers on foreign body giant cells. Bone and Mineral 1994; 27:85-96.

42. Sabokbar A, Kudo O, Itonaga I, Athanasou NA. Role of fibroblasts in osteolysis associated with aseptic loosening. J Bone Joint Surg [Br] 2003; 85-B (Supplement 1):8.

43. Klein DC, Raisz LG. Prostaglandins: stimulation of bone resorption in tissue culture. Endocrinology 1970; 86:1436-1440.

44. Robinson DR, Tashjian AH Jr, Levine L. Prostaglandin-stimulated bone resorption by rheumatoid synovia. A possible mechanism for bone destruction in rheumathoid arthritis. J Clin Invest 1975; 56:1181-1188.

45. Bayne EK, Rupp EA, Limjuco G, Chin J, Schmidt JA. Immunocytochemical detection of interleukin-1 within stimulated human monocytes. J Exp Med 1986; 163:1267-1680.

46. Gowen M, Wood DD, Ihrie EJ, McGuire KB, Graham R, Russel G. An interleukin-1 like factor stimulates bone resorption in vitro. Nature 1983; 306:378-380.

47. Heath JK, Saklatava J, Meikle MC, Atkinson SJ, Reynolds JJ. Pig interleukin-1 (catabolin) is a potent stimulator of bone resorption in vitro. Calcif Tissue Int 1985; 37:95-97.

48. Pfeilschifter J, Chenu C, Bird A, Mundy GR, Roodman GD. Interleukin-1 and tumour necrosis factor stimulate the formation of human osteoclasts like cells in vitro. J Bone Miner Res 1989; 4:113-118.

49. Manolagas SC. Role of cytokines in bone resorption. Bone 1995; 17(Supplement 2):63S-67S.

50. Al-Saffar N, Revell PA. Interleukin-1 production by activated macrophages surrounding loosened orthopaedic implants: a potential role in osteolysis. Br J Rheumatol 1994; 33:309-316.

51. Kim KJ, Chiba J, Rubash HE. In vivo and in vitro analysis of membranes from hip prosthesis inserted without cement. J Bone Joint Surg [Am] 1994; 76-A:172-179.

52. Clark SC. Interleukin-6. Multiple activities in regulation of the hematopoeitic and immune systems. In: Sehgal PB, Grieninger G, Tosato G, eds. Regulation of the acute phase and immune responses: interleukin 6. New York: New York Academy of Sciences 1989; 557:438-443.

53. Durum SK, Oppenheim JJ. Macrophage derived mediators: interleukin-1, tumour necrosis factor, interleukin-6, interferon and related cytokines. In: Paul WE ed Fundamental Immunology. New York: Raven Press Ltd 1989:636-661.

54. Ishimi Y, Miyaura C, Jin CH et al. IL-6 is produced by osteoblasts and induces bone resorption. J Immunol 1990; 145:3297-3303.

55. Conlon K Lloyd A, Chattophadyay U, Lukacs N, Kunkel S, Schall T, Taub D, Morimoto C, Osborne J, Oppenheim J et al. CD-8+ and CD-45RA+ human peripheral blood lymphocytes are potent sources of macrophage inflammatory protein 1a, interleukin-8 and RANTES. Eur J Immunol 1995; 25:751-756.

56. Kristensen MS, Paludan K, Larsen CG, Zachariae CO, Deleuran BW, Jensen PK, Jorgensen P, Thestrup-Pedersen K. Quantitative determination of IL-1 alpha induced IL-8 mRNA levels in cultured human keratinocytes, endothelial cells and monocytes. J Invest Dermatol 1991; 97:506-510.

57. Fuller K, Owens JM, Chambers TJ. Macrophage inflammatory protein 1 alpha and IL-8 stimulate the motility but suppress the resorption of isolated rat osteoclasts. J Immunol 1995; 154:6065-6072.

58. Sabokbar A, Rushton N. Role of inflammatory mediators and adhesion molecules in the pathogenesis of aseptic loosening in total hip arthroplasties. J Arthroplasty 1995; 10:810-816.

59. Lassus J, Waris V, Xu JW, Li TF, Hao J, Nietosvaara Y, Santavirta S, Konttinnen Y. Increased Interleukin-8 (IL-8) expression is related to aseptic loosening of total hip replacement. Arch Ortho Trauma Surg 2000; 120:328-332.

60. Elias JA, Tang W, Horrowitz MC. Cytokine and hormonal stimulation of human osteosarcoma interleukin-11 production. Endocrinology 1995; 136:489-498.

61. Girasole G, Passeri G, Jikka RL Manolagas SC. Interleukin-11: a new cytokine critical for osteoclasts development. J Clin Invest 1994; 93:1516-1524.

62. Xu JW, Li TF, Partsch G, Ceponis A, Santavirta S, Konttinen YT. Interleukin-11 (IL-11) in aseptic loosening of total hip replacement (THR). Scand J Rheumatol 1998; 27:363-367.

63. Introduction to cytokines. In: AP Read & T Brown, ed. Cytokines. Bios Scientific Publishers Limited 1991.

64. Bertolini DR, Nedwin GE, Brigman TS, Smith TS, Smith DD, Mundy GR. Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. Nature 1986; 319:516-518.

65. Jiranek WA, Machado M, Jasty M, Jevsevar D, Wolfe HJ, Goldring SR, Goldberg MJ, Harris WH. Production of cytokines around loosened cemented acetabular components: analysis with immunohistochemical techniques and in situ hybridization. J Bone Joint Surg [Am] 1993; 75-A:863-879.

66. Kong YY, Yoshida H, Sarosi I, Tan HL, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeman A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph node organogenesis. Nature 1999; 397:315-323.

67. Haynes DR, Crotti TN, Potter AE, Loric M, Atkins GJ, Howie DW, Findlay DM. The osteoclstogenic molecules RANKL and RANK are associated with periprosthetic osteolysis. J Bone Joint Surg [Br] 2001; 83-B:902-911.

68. Itonaga I, Sabokbar A, Murray DW, Athanasou NA. Effect of osteoprotegerin ligand on osteoclast formation by arthroplasty membrane derived macrophages. Ann Rheum Dis 2000; 59:26-31.

69. Stanley ER, Guilbert LJ, Tushinki RJ, Bartelmez SH. CSF-1 – a mononuclear phagocyte lineage-specific hemopoetic growth factor. J Cell Biochem 1983; 21:151-159.

70. Rambaldi A, Young DC, Griffin JD. Expression of the M-CSF (CSF-1) gene by human monocytes. Blood 1987; 69:1409-1413.

71. Stern PH, Krieger NS, Nissenson RA, Williams RD, Winkle ME, Derynck R et al. Human transforming growth factor alpha stimulates bone resorption in vitro. J Clin Invest 1985; 76:2016-2019.

72. Tashjian Jr AH, Levine L. Epidermal growth factor stimulates prostaglandin production and bone resorption by epidermal growth factor. Endocrinology 1980; 107:270-273.

73. Xu JW, Li TF, Waris E, Alberty A, Santavirta S, Konttinen YT. Expression of epidermal growth factor and transforming growth factor-alpha in interfacial membranes retrieved at revision total hip arthroplasty. Ann Rheum Dis 2000; 59:822-827.

74. Ross R, Raines EW, Browen-Pope DF. The biology of platelet derived growth factor. Cell 1986; 46:155-169.

75. Matfin G. Cytokines – role in normal bone and pathological states. J Orthop Rheumatol 1992; 5:125-134.

76. Xu JW, Konttinen YT, Li TF, Waris V, Lassus J, Matucci-Cerinic M, Sorsa T, Santavirta S. Production of platelet derived growth factor in aseptic loosening of total hip replacement. Rheumatol Int 1998; 17:215-221.

77. Pollice PF, Silverton S, Horrowitz SM. PMMA-stimulated macrophages increase rat osteoblast precursor recruitment through their effect on osteoblasts in vitro. J Orthop Res 1995; 13:325-334.

78. Horrowitz SM, Purdon MA. Mechanisms of cellular recruitment in aseptic loosening of prosthetic implant. Calcif Tissue Int 1995; 57:301-305.

79. Horrowitz SM, Gonzales JB. Effects of polyethylene on macrophages. J Orthop Res 1997; 15:50-56.

80. Horrowitz SM, Doty SB, Rapuano BP, Lane JM, Burstein AH. Role of the macrophage and the osteoblast in aseptic loosening. Calcif Tissue Int 1994; 54:320-324.

81. Horrowitz MC, Jilka RL. Colony stimulating factors and bone remodelling. In: Gowen M (ed) Cytokines and bone metabolism. CRC press, Boca Raton,1992: pp 194-201.

82. Lowik CWGM. Differentiation inducing factors: leukaemia inhibitory factor and interleukin-6. In: Gowen M (ed) Cytokines and bone metabolism. CRC press, Boca Raton, 1992: pp 300-324.

83. Kadoya Y, Revell PA, Al-Saffer N, Kobayashi A, Scott G, Freeman MAR. Bone formation and bone resorption in failed total joint arthroplasties: histomorphometric analysis with histochemical and immunohistochemical technique. J Orthop Res 1996; 14:473-482.

84. Greenfield EM, Bi Y, Ragab AA, Goldberg VM, Van De Motter RR. The role of osteoclast differentiation in aseptic loosening. J Orthop Res 2002; 20:1-8.

85. The matrix metalloproteinase family. In: Parks WC & Mecham RP, ed. Matrix Metalloproteinases. Academic Press 1998.

86. Woessner JF. Matrix metalloproteinases and their inhibitors in connective tissue remodelling. FASEB J 1991; 5:2145-2154.

87. Baron R, Chakraborty M, Chatterjee D et al. Biology of the osteoclast. In: Mundy GR, Martin TJ, eds. Physiology and Pharmacology of bone. New York: Springer 1993.

88. Sims N, Baron R. Bone cells and their function. In: Ernesto canalis, ed. Skeletal Growth Factors. Lippincott Williams & Wilkins, Philadelphia 2000.

89. Etherington DJ. The purification of bovine cathepsin B1 and its mode of action on bovine collagens. Biochem J 1974; 137:547-57.

90. Kirschke H, Kembhavi AA, Bohley P, Barrett AJ. Action of rat liver cathepsin L on collagen and other substrates. Biochem J 1982; 201:367-372.

91. Etherington DJ, Birkedal-Hansen H. The influence of dissolved calcium salts on the degradation of hard tissue collagens by lysosomal cathepsins. Collagen Rel Res 1987; 7:185-199.

92. Maciewicz RA, Etherington DJ. A comparison of four cathepsins (B, L, N and S) with collagenolytic activity from rabbit spleen. Biochem J 1988; 256:433-440.

93. Eeckhout Y, Delaissé JM, Ledent P, Vaes G. The proteinases of bone resorption. In: Glauert A, ed. The Control of Tissue Damage. Amsterdam; Elsevier 1998.

94. Vaes G. Cellular biology and biochemical mechanism of bone resorption. A review of recent developments on the formation, activation, and mode of action of osteoclasts. Clin Ortho Relat Res 1988; 231:239-271.

95. Delaissé JM, Eeckhout Y, Vaes G. Inhibition of bone resorption in culture of by inhibitors of thiol preteinases. Biochem J 1980; 192:365-368.

96. Vaes G. The release of collagenase as an inactive proenzyme by bone explants in culture. Biochem J 1972; 126:275-289.

97. Eeckhout Y, Delaissé JM, Vaes G. Direct extraction and assay of bone tissue collagenase and its relation to parathyroid hormone induced bone resorption. Biochem J 1986; 239:793-796.

98. Sakamoto S, Sakamoto M. Biochemical and immunohistochemical studies on collagenase in resorbing bone in tissue culture. A novel hypothesis for mechanism of bone resorption. J Period Res. 1982; 17:523-526.

99. Delaissé JM, Eeckhout Y, Neff L, Francois-Gillet C, Henriet P, Su Y et al. (Pro)collagenase (MMP-1) is present in rodent osteoclasts and in the underlying bone resorbing compartment. J Cell Sci 1993; 106:1071-1082.

100. Delaissé JM, Eeckhout Y, Sear C, Galloway A, McCullach K, Vaes G. A new synthetic inhibitor of mammalian tissue collagenase inhibits bone resorption in culture. Biochem Biophys res Commun 1985; 33:483-490.

101. Case JP, Sano H, Lafyatis R, Remmers EF, Kumkumian GK, Wilder RL. Transin stromelysin expression in synovium of rats with experimental erosive arthritis. In situ localization and kinetics of expression of the transformation –associated metalloproteinase in euthymic and athymic Lewis rats. J Clin Invest 1989; 84:1731-1740.

102. Okada Y, Naka K, Kawamura K, Matsumoto T, Nakanishi I, Fujimoto N, Sato H, Seiki M. Localization of marix metalloproteinase 9 in osteoclasts: Implications for bone resorption. Lab Invest 1995; 72:311-322.

103. Takagi M, Konttinen YT, Santarvirta S, Sorsa T, Eisen AZ, Nordsletten L, Suda A. Extracellular matrix metalloproteinases around loose total hip prosthesis. Acta Orthop Scand 1994; 65:281-286.

104. Yokohama Y, Matsumoto T, Hirakawa M, Kuroki Y, Fujimoto N, Imai K, Okada Y. Production of matrix metalloproteinases at the bone implant interface in loose total hip replacements. Lab Invest 1995; 73:899-911.

105. Takei I, Takagi M, Santarvirta S, Ida H, Ishii M, Ogino T, Ainola M, Konttinen YT. Messenger ribonucleic acid expression of 16 matrix metalloproteinases in bone-implant interface tissues of loose artificial hip joints. J Biomed Mater Res 2000; 52:613-620.

106. Kavanagh BF, Ilstrup DM, Fitzgerald RH. Revision total hip arthroplasty. J Bone Joint Surg [Am] 1985; 67A:517-526.

107. Pellicci PM, Wilson Jr PD, Sledge CB, Salvati EA, Ranawat CS, Poss R, Callaghan JJ. Long term results of revision total hip replacement. A follow up report. J Bone Joint Surg [Am] 1985; 67A:513-516.

108. Stromberg CN, Herberts P. A multicenter 10-year study of cemented revision total hip arthroplasty in patients younger than 55 years old. A follow up report. J Arthrop 1994; 9(6);595-601.

109. Pandey R, Quinn JM, Sabokbar A, Athanasou NA. Bisphosphonate inhibition of bone resorption induced by particulate biomaterial-associated macrophages. Acta Orthop Scand 1996; 67:221-228.

110. Horrowitz SM, Algan SA, Purdon MA. Pharmacologic inhibition of particulate induced bone resorption. J Biomed Res 1996; 31:91-96.

111. Shanbhag AS, Hasselman CT, Rubash HE. Inhibition of wear debris mediated osteolysis in a canine total hip replacement model. Clin Orthop 1997; 344:33-43.

112. Sato M, Grasser W, Endo N, Akins R, Simmons H. Bisphosphonates action: Alendronate localization in rat bone and effects on osteoclast ultrastructure. J Clin Invest 1991; 88:2095-2105.

113. Rodan GA, Fleisch HA. Bisphosphonates: Mechanisms of action. J Clin Invest 1996; 97:2692-2696.

114. Jiranek WA, Machado M, Jasty M, Jevsevar D, Wolfe HJ, Goldring SR, Goldberg MJ, Harris WH. Production of cytokines around loosened cemented acetabular components: analysis with immunohistochemical techniques and insitu hybridization. J Bone Joint Surg [Am] 1993; 75A:863-879.

115. Schwarz EM, Benz EB, Lu AP, Goater JJ, Mollano AV, Rosier RN, Puzas JE, O'Keefe RJ. Quantitative small animal surrogate to evaluate drug efficacy in preventing wear debris induced osteolysis. J Orthop Res 2000; 18:849-855.

116. Pollice PF, Rosier RN, Looney RJ et al. Oral pentoxyfyline inhibits release of tumour necrosis factor-alpha from human peripheral blood monocytes: a potential treatment for aseptic loosening of total joint components. J Bone Joint Surg [Am] 2001; 83A:1057-1061.

117. Doxycycline. In: Dollery C, ed. Therapeutic drugs. 2nd edition. Churchill Livingstone 1999.

118. Golub LM, Lee H-M, Lehrer G, Nemiroff A, McNamara TF, Kaplan R, Ramamurthy NS. Minocycline reduces gingival collagenolytic activity during diabetes: preliminary observations and a proposed new mechanism of action. J Peridont Res 1983; 18:516-526.

119. Golub LM, Ramamurthy NS, McNamara TF, Greenwald RA, Rifkin BR. Tetracyclines inhibit connective tissue breakdown: New therapeutic implications for an old family of drugs. Crit Rev Oral Biol Med 1991; 2:297-322.

120. Vernillo AT, Ramamurthy NS, Golub LM, Rifkin BR. The non antimicrobial properties of tetracycline for the treatment of periodontal disease. Curr Opinion Peridont 1994; 111-118.

121. Burns F, Stack M, Gray R, Paterson C. Inhibition of purified collagenase from alkaline burned rabbit corneas. Invest Opthalmol Vis Sci 1989; 30:1569-1575.

122. Gomes BC, Golub LM, Ramamurthy NS. Tetracyclines inhibit parathyroid hormone-induced bone resorption in organ culture. Experientia 1984; 40: 1273-1275.

123. Donahue HJ, Iijima K, Goligorsky MS, Rubin CT, Rifkin BR. Regulation of cytoplasmic calcium concentration in tetracycline treated osteoclasts. J bone Miner Res 1992; 7:1313-1318.

124. Rifkin BR, Vernillo AT, Golub LM. Blocking periodontal disease progression by inhibiting tissue-destructive enzymes: a potential therapeutic role for tetracyclines and their chemically modified analogs. J Peridontol 1993; 64:819-827.

125. Rifkin BR, Ramamurthy NS, Gay CV. Effects of tetracyclines on acid production by isolated osteoclasts [abstract]. J Dent Res 1992; 71:1038.

126. Greenwald RA, Golub LM, Lavietes B, Ramamurthy NS, Laskin R, Gruber B. Tetracyclines inhibit human synovial collagenase in vivo and in vitro. J Rheumatol 1987; 14:28-32.

127. Golub LM, Ramamurthy NS, Kaneko H, Sasaki T, Rifkin BR, McNamara TF. Tetracycline administration prevents diabetes induced osteopenia in the rat: initial observations. Res Commun Chem Pathol Pharmacol 1990; 68:27-40.

128. Moghadasian MH. Clinical pharmacology of 3-Hydroxy-3-methylglutaryl Co-enzyme A reductase inhibitors. Life Sci 1999; 65:1329-1337.

129. Fluvastatin. In: Dollery C, ed. Therapeutic drugs. 2nd edition. Churchill Livingstone 1999.

130. Corsini A, Bellosta S, Baetta R, Fumagalli R, Paoletti R, Bernini F. New insights into the pharmacodynamic and pharmacokinetic properties of statins. Pharmmacology & Therapeutics 1999; 84:413-428.

131. Dollery CM, McEvan JR, Henney AM. Matrix metalloproteinases and cardiovascular disease. Circ Res 1995; 77:863-868.

132. Falk E, Shah PK, Fuster V. Coronary plague disruption. Circulation 1995; 92:657-671.

133. Bellosta S, Via D, Canavesi M, Pfister P, Fumagali R, Paoletti R, Bernini F. HMG-CoA reductase inhibitors reduce MMP-9 secretion by macrophages. Arterioscler Thromb Vasc Biol 1998; 18:1671-1678.

134. Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G et al. Stimulation of bone formation in vitro and in rodents by statins. Science 1999; 286:1946-1949.

135. Maeda T, Matsunuma A, Kawane T, Horiuchi N. Simvastatin promotes osteoblast differentiation and mineralization in MCT3T3-E1 cells. Biochem Biophys Res Comm 2001; 280:874-877.

136. Nuti R, Martini G, Righi G, Frediani B, Turchetti V. Comparison of total-body measurements by dual energy X-ray absorptiometry and dual photon absorptiometry. J Bone Miner Res 1991; 6: 681-687.

137. Parvori R, DeVecchi E, Fermo I, et al. Total urinary hydroxyproline determined with rapid and simple high performance liquid chromatography. Clin Chem 1992; 38:407-411.

138. Moro L, Modricky C, Stagni N, Vittur F, de Bernard B. High performance liquid chromatographic analysis of urinary hydroxylysyl glycosides as indicators of collagen turnover. Analyst 1984; 109:1621-1622.

139. Segrest JP, Cunningham LW. Variation in human urinary O-hydroxylysyl glycosides levels and their relationship to collagen metabolism. J Clin Invest 1970; 49:1497-1509.

140. Eyre DR. Bone biomarkers as tools in osteoporosis management. Spine 1997; 22:17S-24S.

141. Ek-Rylander B, Flores M, Wendel M, Heinegard D, Anderrsson G. Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. J Biol Chem 1994; 269:14853-14856.

142. Lau KHW, Orishi T, Wargedal JE, Singer FR, Baylink DJ. Characterization and assay of tartrate-resistant acid phosphatase activity in serum: potential use to assess bone resorption. Clin Chem 1987; 33:454-562.

143. Hattersley G, Chambers TJ. Generation of osteoclastic function in mouse bone marrow cultures: multinuclearity and tartrate-resistant acid phosphatase are unreliable markers for osteoclastic differentiation. Endocrinology 1989; 124:1689-1696.

144. Demers LM, Kleerekoper M. Recent advances in biochemical markers of bone turnover (editorial). Clin Chem 1994; 40:1994-1995.

145. Robins SP, Stewart P, Astbury C, Bird HA. Measurement of the cross linking compound, pyridinoline, in urine as an index of collagen degradation. Ann Rheum Dis 1986; 45:969-973.

146. Black D, Duncan A, Robins SP. Quantitative analysis of the pyridinium cross-link of collagen in urine using ion paired reverse phase high performance liquid chromatography. Anal Biochem 1988; 169:197-203.

147. Eyre DR. Collagen cross-linking amino acids. Methods Enzymol 1987; 144:115-139.

148. Beardsworth LJ, Eyre DR, Dickson IR. Changes with age in the urinary excretion of lysyl- and hydroxylysyl-pyridinoline: Two new markers of bone collagen turnover. J Bone Miner Res 1990; 5:671-676.

149. Uebelhart D, Schlemmer A, Johansen JS, Gineyts E, Christiansen C, Delmas PD. Effect of menopause and hormone replacement therapy on the urinary excretion of pyridinium cross-links. Clin Endocrinol Metab 1991; 72:367-373.

150. Eyre DR. The specificity of collagen cross-links as markers of bone and connective tissue degradation. Acta Ortho Scand Suppl 1995; 266:166-170.

151. Seibel MJ, Robins SP, Bilezikian JP. Urinary pyridinium cross-links of collagen: Specific markers of bone resorption in metabolic bone disease. Trends Endocrinol Metab 1992; 3:263-270.

152. Hanson DA, Eyre DR. Molecular site specificity of pyrodinoline and pyrole cross-links in type 1 collagen of human bone. J Biol Chem 1996; 271:26508-26516.

153. Hanson DA, Weis M-AE, Bollen A-M, Maslan AL, Singer SR, Eyre DR. A specific immunoassay for monitoring human bone resorption: Quantitation of type 1 collagen cross-linked N-Telopeptides in urine. J Bone Miner Res 1992; 7:1251-1258.

154. Eyre DR, Van Ness K, Koob TJ. Quantitation of hydroxyl pyridinium crosslinks in collagen by high performance liquid chromatography. Anal Biochem 1984; 137:380-388.

155. Apone S, Lee MY, Eyre DR. Osteoclasts generate cross-linked collagen N-Telopeptides (NTx) but not free pyridinolines when cultured on human bone. Bone 1997; 21:129-136.

156. Gertz BJ, Shao P, Hanson DA, et al. Monitoring bone resorption in early post menopausal women by an immunoassay for cross-linked collagen peptides in urine. J Bone Miner Res 1994; 9:135-142.

157. Clemens JD, Herrick MV, Singer FR, Eyre DR. Evidence that serum NTx (collagen-type 1 N telopeptides) can act as an immunochemical marker of bone resorption. Clin Chem 1997; 43:2058-2063.

158. Risteli J, Elomaa I, Niemi S, Novamo A, Risteli L. Radioimmunoassay for the pyrodinoline cross-linked carboxy-terminal telopeptide of type 1 collagen: A new marker of bone degradation. Clin Chem 1993; 39:635-640.

159. Bonde M, Qvist P, Fledelius C, Riis BJ, Christiansen C. Immunoassay for quantifying type 1 collagen degradation products in urine evaluated. Clin Chem 1994; 40:2022-2025.

160. Bonde M, Qvist P, Fledelius C, Riis BJ, Christiansen C. Applications of an enzyme immunoassay for a new marker of bone resorption (CrossLaps): Follow-up on hormone replacement therapy and osteoporosis risk assessment. J Clin Endocrinol Metab 1995; 80:864-868.

161. Garnero P, Gineyts E, Riou JP, Delmas PD. Assessment of bone resorption with a new marker of collagen degradation in patients with metabolic disesase. J Clin Endocrinol Metab 1994; 79: 780-785.

162. Risteli J, Risteli L. Assays of type procollagen domains and collagen fragments: Problems to be solved and future trends. Scand J Clin Lab Invest 1997; 57(Suppl):105-113.

163. Schneider U, Breusch SJ, Termath S, Thomsen M, Brocai DRC, Nietherd F-U, Karsperk C. Increased urinary crosslink levels in aseptic loosening of total hip arthroplasty. J Arthrop 1998; 13:687-692.

164. Antoniou J, Huk O, Zukor D, Eyre D, Alini M. Collagen crosslinked N-Telopeptides as markers for evaluating particulate osteolysis: Preliminary study. J Orthop Res 2000; 18:64-67.

165. Apone S, Lee MY, Eyre DR. Osteoclasts generate cross-linked collagen N-Telopeptides (NTx) but not free pyridinolines when cultured on human bone. Bone 1997; 21:129-136.

166. Reynolds JJ, Dingle JT. A sensitive in vitro method for studying the induction and inhibition of bone resorption. Calcif Tissue Res 1970; 4:339-349.

167. Sabokbar A, Fujikawa Y, Neale S, Murray DW, Athanasou A. Human arthroplasty derived macrophages differentiate into osteoclastic bone resorbing cells. Ann Rheumat Dis 1997; 56:414-420.

168. Danks L, Sabokbar A, Hirayama T, Gundle R, Athanasou NA. Osteoclast precursors in periprosthetic tissues and arthritic synovium are CD14+ (abstract). BORS meeting, London, October 2000.

169. Doyle A, Griffiths JB. Haemocytometer cell counts and viability study. In: Cell and tissue culture for medical research. John Wiley & Sons Ltd, 2000:12-16.

170. Scheven BAA, Milne JS, Robins JS. A novel culture system to generate osteoclasts and bone resorption using porcine bone marrow cells: role of M-CSF. Biochem Biophys Res Comm 1997; 231:231-235.

171. Greenwald RA, Golub LM, Ramamurthy S, et al. In vitro sensitivity of the three mammalian collagenases to tetracycline inhibition: relationship to bone and cartilage degradation. Bone 1998; 22:33-38.

172. Ramamurthy S, Bain S, Liang CT, et al. A combination of subtherapeutic doses of chemically modified doxycycline (CMT-8) and bisphosphonate (clodronate) inhibits bone loss in ovariectomized rat: a dynamic histomorphometric and gene expression study. Curr Med Chem 2001; 8:295-303.

173. Green J. The physiochemical structure of bone: cellular and non-cellular elements. Miner Electrolyte Metab 1994; 20:7-15.

174. Vernillo AT, Ramamurthy NS, Golub LM, Rifkin BR. The non antimicrobial properties of tetracycline for the treatment of periodontal disease. Curr Opin Peridontal 1994; 111-118.

175. Pascale D, Gordon J, Lamster I, Mann P, Seiger M, Arndt W. Concentration of doxycycline in human gingival fluid. J Clin Peridont 1986; 13:841-844.

176. Chowdhury MH, Moak SA, Rifkin BR, Greenwald RA. Effect of tetracyclines which have metalloproteinase inhibitory capacity on basal and heparin stimulated bone resorption by chick osteoclasts. Agents Actions 1993; 40:124-128.

177. Leung A, Scammell B, Lyons A, Czachur M, Gilbert J, Freedholm D, Malbecq W, Miller C, Carr A, Checkley L. Alendronate prevents periprosthetic bone loss – 2 year results. Arthritis Rheum 1999; 42(Suppl):S270.

178. Ulrich-Vinther M, Carmody EE, Goater JJ, Søballe K, O'Keefe RJ, Schwarz EM. Recombinant adeno-associated virus-mediated osteoprotegerin gene therapy inhibits wear debris osteolysis. J Bone Joint Surg [Am] 2002; 84A:1405-1412.